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Patricia Ann McQueney
Lehigh University

AUTHOR:

McQueney, Patricia A.

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ANALYSIS OF R68.45 AND R68.45-*met* R-PRIMES BY PHYSICAL AND GENETIC CRITERIA

by

Patricia Ann McQueney

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Professor Steven Krawiec
Thesis advisor

Professor Michael R. Kuchka

Professor Jeffrey A. Sands
Chairman of Department

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ABSTRACT

The IncP-1 plasmid R68.45 possesses many attributes which make it attractive for use in bacterial genetics. These attributes include a broad-host-range phenotype, enhanced chromosome mobilization, resistance to several antibiotics and the ability to form prime plasmids. Despite the attractive features, the plasmid is not used extensively in bacterial genetics. The use of the plasmid is limited by two major problems: i) the difficult isolation and characterization of the plasmid and ii) plasmid instability. During this study, techniques have been adapted which allow the consistent isolation of the plasmid in high yields. Also, pulsed field gel electrophoresis has been introduced as a method to examine the physical characteristics of the plasmid.

Interspecific matings between *P. putida* and *P. aeruginosa* were used to generate R68.45-*met* R-primes in *P. aeruginosa* methionine auxotrophs. The conversion of methionine auxotrophy to prototrophy upon the receipt of the plasmid from the donor provided genetic evidence of the existence of R-*met* primes. Plasmid isolation provided physical evidence for the presence of the primes. The primes were found to be stable in the *P. aeruginosa* host.

Following the transfer of the R-primes to *E. coli* B through intergeneric matings, the primes were analyzed on

genetic and physical levels. The genetic analysis revealed that the plasmids had lost the ability to express the tetracycline resistance marker and were unable to cotransfer plasmid and chromosomal markers. These results suggest that the R-prime had become unstable during or after the transfer to the new host.

Restriction analysis of the R-primes isolated from the *E. coli* B transconjugants revealed that the plasmids had lost a segment of DNA in the *Hind*III/*Sal*I fragment. The *Hind*III/*Sal*I fragment is associated with the $(IS21)_2$ sequence. The loss of the IS21 sequence would revert R68.45 to the structure of its parental plasmid. This result could imply that there is a basic genetic organization associated with stable plasmids.

INTRODUCTION

Many bacteria of diverse types harbor plasmids. Plasmids are generally defined as non-essential extrachromosomal, double-stranded, circular DNA molecules which exist autonomously in bacterial cells. Plasmid DNA encodes a variety of phenotypic properties exhibited by the host organism. These properties include: resistance to antibiotics, heavy metal cations, and anions; metabolic functions; and properties contributing to pathogenicity or symbiosis.

Plasmids of the incompatibility group P-1 may play an increasingly important role in the genetic analysis of gram-negative bacteria for which conjugation systems have not previously existed or which are unsatisfactory. The IncP-1 plasmid R68.45 (figure 1) has many attributes which make it a valuable tool in the genetic analysis of bacterial species. These attributes include its broad-host-range; its enhanced chromosome mobilizing phenotype (ECM); its selectability (due to the existence of antibiotic resistance factors for ampicillin, carbenicillin, kanamycin, and tetracycline); and its demonstrated ability to form plasmid-chromosome hybrids, known as prime plasmids.

The molecular mechanisms behind the broad-host-range

and the ECM phenotypes have been examined extensively. However, the mechanism(s) of R-prime formation remains largely unknown. In this study, *Pseudomonas putida* R-primes were generated in *P. aeruginosa* and subsequently analyzed by physical and genetic methods.

Broad-host-range of R68.45

Many plasmids of gram-negative bacteria are only maintained stably in a limited range of closely related species. Others are maintained in a diverse range of bacterial species and are termed broad-host-range plasmids. IncP-1 plasmids, including R68.45, are broad-host-range plasmids. The promiscuous nature of IncP-1 plasmids requires two properties: replication and maintenance. The genetic mechanisms responsible for these two properties involve a rather complex series of coordinated operons and is not fully understood (Thomas and Helinski, 1989). The plasmid's origin of vegetative replication (*ori* V) and the product of the trans-acting replication gene *trf*A are the minimal genomic segments required for the promiscuous replication of the plasmid (Schmidhauser and Helenski, 1985). Contributing to the broad-host-range is the fact that particular segments of DNA within the *ori*V are differentially required for the replication of the plasmid in various bacterial hosts (Nash and Krishnapilla, 1987).

The protein product(s) of the plasmid-encoded *trf*A gene initiate replication at the *ori*V. The *trf*A gene produces two polypeptides which function differently in various bacterial species to provide a degree of flexibility in the interaction of the plasmid replication system and host proteins (Shingler and Thomas, 1989).

The *ori*V and *trf*A are insufficient for the stable maintenance of IncP-1 plasmids. The broad-host-range maintenance requirements are encoded by multiple functions, and the requirement for these varies among gram-negative bacteria (Schmidhauser and Helinski, 1985). The regions involved in the stable maintenance have been reviewed by Thomas and Helinski (Thomas and Helinski, 1989). These regions include the *kil* (kill) and *kor* (*kil*-override) genes and the *inc*C region. The determinants of the *kor*A-*kor*B operon, namely *kor*A, *kor*B, and *inc*C alter plasmid stability; however, the effects are not uniform in all bacterial hosts (Schmidhauser et al., 1989). The *kil* genes are part of the *trf*A operon and are either host lethal or inhibitory to plasmid maintenance (Figurski et al., 1982). The products of two genes, *kor*A and *kor*B, negatively regulate the *trf*A promoter (Shingler and Thomas, 1984). *kor*A and *kor*B have multiple regulatory functions besides regulation of *trf*A including, autoregulation (Smith et al., 1984), and regulation of the host lethal *kil* genes (Figurksi et al.,

1982). The *incC* region is involved in the incompatibility function (Schmidhauser et al., 1985). The recently discovered *korF* gene is also implicated to play a role in the promiscuous phenotype, although the exact role remains unknown (Thomas et al. 1990). In summary, there are different requirements for stable plasmid maintenance in a variety of hosts, and the ability of IncP-1 plasmids to adapt to the variety of intracellular environments requires the complex coordination of many plasmid as well as genomic sequences.

Chromosome mobilization

The phenotypic ability of plasmids to transfer the host chromosome has been denoted chromosome mobilization ability (Cma). Cma permits the study of bacterial genetics by facilitating genetic mapping and allowing for the isolation of recombinants from unrelated species. Cma is best understood in and was initially described for the F plasmid of *Escherichia coli*. Now it has become apparent that Cma is a common phenotype among plasmids found in widely different genera of bacteria. Among plasmids which express the Cma phenotype are the IncP-1 plasmids, including R68.45. R68.45 has been described as possessing enhanced chromosome mobilization ability (ECM) due to its ability to transfer the chromosomes from diverse species with high efficiency.

Chromosome transfer by R68.45 has been described in several species of *Pseudomonas* (Haas and Holloway, 1978; Martinez and Clark, 1975; Tatara and Goodwin, 1983), *Erwina* (Chatterjee, 1980), *Rhizobia* (Beringer et al., 1978; Johnston and Beringer, 1977; Kondorosi et al., 1977; McLaughlin and Ahmad, 1986; Megias et al., 1982; Yu et al., 1981), and specifically in *E. coli* (Riess et al., 1980), *Bordella pertussi* (Smith et al., 1986), *Agrobacterium tumefaciens* (Hamada et al., 1979; Bryan et al., 1982), and *Rhodopseudomonas sphaeroides* (Sistrom, 1977). R68.45 also demonstrates the ability to mobilize non-conjugative plasmids. Plasmid mobilization has been shown to occur in *R. trifoli* (Scott and Ronson, 1982), and *E. coli* (Riess et al., 1983).

The best example of plasmid-mediated Cma for which the molecular mechanism of chromosome transfer has been studied extensively has been that of F in *E. coli*. F stably integrates into the chromosome at a variety of sites forming Hfr strains and, thus, creates specific transfer origins and directions of transfer (*ie.*, polarized transfer). The DNA transferred during conjugation is single stranded with DNA replication commencing from the origin of transfer (*oriT*).

The actual mechanism of Cma mediated by R68.45 is not known. However, there is little evidence that R68.45 stably integrates into the chromosome to form an Hfr-like

derivative of R68.45 (Haas and Holloway, 1976; Kiss et al., 1980; Reimmann et al., 1986). Cma appears to be dependent on the formation of a transient cointegrate between R68.45 and the host chromosome. R68.45 differs from its parental plasmid R68 by a 2.1 kb DNA segment. The extra DNA is in part a repetition of a 2.1 kb segment already present on R68 (Riess et al., 1980; Jacob et al., 1977). The tandem duplication of the 2.1 kb segment on R68.45 has been termed IS21 [=$(IS21)_2$] (Willetts et al., 1981). Spontaneous loss of one IS21 element of $(IS21)_2$ or the entire $(IS21)_2$, results in the loss of Cma (Haas and Riess, 1983; Currier and Morgan, 1982), indicating that the $(IS21)_2$ sequence is essential for the ECM phenotype expressed by R68.45. The molecular basis of chromosome mobilization may be dependent on the transposition properties of $(IS21)_2$ (Willetts et al., 1981) to mediate the formation of a cointegrate between R68.45 and the chromosome. The formation of R68.45-replicon cointegrates has been observed when R68.45 mobilizes non-conjugative plasmids (Scott and Ronson, 1982; Riess et al., 1983) and between replication deficient R68.45 and the chromosome (Reimmann et al., 1988). In the cointegrate, the mobilized plasmid is flanked by single copies of IS21 in direct orientation (Riess et al., 1983). Similar structures were found in the chromosomally integrated plasmids

(Reimmann et al., 1988). During resolution of the cointegrate, the mobilizing plasmid R68.45 loses a copy of IS21 (Riess et al., 1983). These structures support a "cut and paste" model of R68.45 transposition (Reimmann and Haas, 1987) where a transposase preferentially cuts R68.45 between the tandemly repeated IS21's precisely at the inner 3' ends of each IS21 in the $(IS21)_2$ resulting in 5' protrusions (Reimmann and Haas, 1990). It has been proposed that the inner 3' ends are then joined to the 5' ends of the target, thus integrating the entire plasmid into the target replicon (Reimmann and Haas, 1990). Once integrated, R68.45 may transfer the chromosome in a manner similar to the F plasmid of *E. coli* (*ie.*, with the transfer of single stranded DNA commencing at the origin of transfer).

The precise molecular mechanism of $(IS21)_2$ transposition and its involvement in the ECM of R68.45 is slowly coming into focus. The single copy of IS21 in R68 is inactive in terms of possessing transposition properties, whereas the tandem duplication, $(IS21)_2$, in R68.45 is active in transposition (Willetts et al., 1981). The high transpositional activity of $(IS21)_2$ appears to result from two properties of the IS21-IS21 junction. The IS21 transposase acts more readily at the junction than the outer ends of $(IS21)_2$ (Reimmann and Haas, 1987). Additionally,

the presence of the right-hand copy, IS21R, leads to transcription into the left-hand copy, IS21L (Schurter and Holloway, 1986). IS21L has been defined as the IS21 element next to the kanamycin resistance gene on R68.45 (Schurter and Holloway, 1986). The promoter reading into the IS21L spans the IS21-IS21 junction (Reimmann et al., 1989). IS21R contains the -35 region of the promoter and IS21L the -10 region of the promotor (Reimmann et al., 1989). Each IS21 element contains two open reading frames, *ist*A and *ist*B (Reimmann et al., 1989). A single copy of IS21 does not produce IstA and IstB (Reimmann et al., 1989). However, the formation of an active promotor at the IS21-IS21 junction initiates transcription of the *ist*A and *ist*B genes in one of the two IS21 elements (Reimmann et al., 1989). IstA and IstB are both essential for *in vivo* transposition of $(IS21)_2$. However, their precise functions remain unknown (Reimmann et al., 1990). IstA cleaves the IS21-IS21 sequence at the inner 3′ end of the junction producing staggered protrusions, thus suggesting that IstA could serve as the IS21 transposase (Reimmann et al., 1990).

As a reflection of the different mechanisms of Cma mediated by R68.45 and by F, the properties of marker transfer differ between the two plasmids. R68.45 integrates into the chromosome at many sites producing many origins of

transfer located throughout the chromosome (Haas and Holloway, 1978). Due to the large number of integration sites, each chromosomal marker may be mobilized from different origins and may be transmitted in both the clockwise and anticlockwise direction giving the appearance of non-polarized marker transfer (Haas and Holloway, 1978). In contrast, F integrates into few sites and chromosomal transfer proceeds in a polarized way (clockwise or anticlockwise) (Umeda and Ohstubo, 1989). The frequency of transfer of markers located throughout the chromosome mediated by R68.45 is similar due to non-polarized nature of marker transfer (Haas and Holloway, 1978). In contrast, since the transfer mediated by F is in a polarized fashion, markers located near the origin of transfer have a higher frequency of transfer then markers located further from the origin. Related to the many origins of transfer is the fact that R68.45 transfers shorter segments of the chromosome then does F (Haas and Holloway, 1978). Finally, R68.45 recombinants express plasmid markers, whereas, in Hfr recombinants, plasmid markers are rarely inherited (Chatterjee, 1980).

R-primes

Plasmid-chromosome hybrids or plasmid-plasmid hybrids--known as prime plasmids--were first described and are most

extensively examined for the F plasmid of *E. coli*. Similar structures have been isolated from the IncP-1 plasmids. The most widely used IncP-1 plasmid utilized in the formation of R-primes is R68.45. R68.45-primes, containing non-conjugative plasmid genes, have been isolated from *P. putida* (Herrmann et al., 1988) and *R. meliloti* (Banfalvi et al., 1983). R68.45 or R68.45 derived plasmids have also been used to isolate R-primes containing chromosomal inserts from several gram-negative bacteria. R68.44, a less stable derivative of R68 was used to isolate R-primes in *P. putida* (Beeching et al., 1983) and *P. aeruginosa* (Hedges and Jacob, 1977; Hedges and Jacob, 1977b). R68.45 was used to isolate R-primes in *P. aeruginosa* (Holloway, 1978; Morgan, 1982; Shinomiya et al., 1983; Tsuda et al., 1981); *Rhizobium* spp. (Johnston et al., 1978; Johnston et al., 1978b; Kiss et al., 1980; Kowalczuk et al., 1981); and *Klebsiella pneumoniae* (Epsin et al., 1981). pMO61 a kanamycin sensitive derivative or R68.45 was used to isolate R-primes in *P. putida* (Bray et al., 1987) and *Rhizobium* (Nayundu and Rolfe, 1987). Finally, pJB3JI a R68.45 derivative containing a single IS21 sequence was used to generate R-primes in *Bradyrhizobium japonicum* and *R. fredii* (Shah et al., 1989). R-primes have many uses in the study of bacterial genetics including: localization of alleles responsible for a phenotype (Shinomiya et al., 1983; Johnston et al., 1978);

complementation of mutations in a particular chromosomal region to determine linkage relationships among alleles (Epsin et al., 1981); genetic manipulation of genes required for a specific phenotypes (Nayudo and Rolfe, 1987); identification and differentiation of auxotrophic mutations used for strain construction (Morgan, 1982); determination of the *distribution* of alleles (*ie.*, to determine if the genes responsible for a particular phenotype are clustered or non-clustered) (Johnston et al., 1978; Tsudo et al., 1981; Banfalvi et al., 1983); examination of the nature of the regulatory systems that operate when nonhomolgous DNA has the opportunity to be expressed in a foreign bacterial environment (Hedges et al., 1977); and as *in vivo* cloning vectors (Kowalczuk et al., 1981).

Despite the many uses of primes in the examination of bacterial genetics the mechanisms of their formation remains largely unknown. The most extensive studies concerning the mechanism of prime plasmid formation have been done with the F plasmid of *E. coli*. The formation of a prime plasmid involves two steps: the interaction of the plasmid with the target DNA, and excision from the target DNA. F-primes are mainly formed from Hfr strains. Integration of F into the chromosome, to form the Hfr, may occur via homologous recombination between insertion sequences on the plasmid and identical insertion sequences on the chromosome (Hu et al.,

1975; Ohtsubo and Hsu, 1978; Virolle et al., 1983; Umeda and Ohtusbo, 1989). However, some Hfrs are cointegrates formed by replicative transposition of an element of F, the gamma-delta segment, to a chromosomal region not containing insertion elements (Umeda and Ohtusbo, 1989). After integration of F into the chromosome, the prime may be formed by several different excision methods; by aberrant excision (Scaife, 1967); by transpositional mechanism involving the gamma-delta transposon (Hadley and Deonier, 1980); and by homologous recombination between insertion elements located on either the plasmid or the chromosome (Deonier et al., 1977; Ohtsubo and Hsu, 1978b; Timmons, 1983). Two types of excision may occur; excision by homologous recombination between insertion elements on the integrated F and the same insertion element on the chromosome; or excision by homologous recombination between insertion elements on chromosomal sequences on both sides of the integrated F (Umeda and Ohtsubo, 1989).

Much less is known about the mechanism(s) of R-prime formation in the Inc-P plasmids (*ie.*, R68.45). It is clear that the mechanism differs form that of F, since R68.45 does not stably integrate into the chromosome to form an Hfr-like strain. One prerequisite for the formation of an R-prime is the ability of R68.45 to interact with the host chromosome. Currently there are two disparate views of how R68.45

initially interacts with the target DNA: 1) the formation of R-primes requires the $(IS21)_2$ sequence; or 2) R-prime formation does not depend on the IS21 sequence. It has been suggested that the IS21 sequence interacts with sequences on the chromosome to form an unstable integrated plasmid (Leemans et al., 1980; Riess et al., 1980). The proposed mechanism of this interaction has been described previously. If the IS21 does mediate the interaction of R68.45 and the target DNA in the formation a prime, then it would be expected that the inserted DNA molecule would be flanked by two single IS21 copies. Structures consistent with this prediction have been observed in several cases. Physical analysis of a pMO61 R-prime in *P. putida* (Bray et al., 1987) and a R68.45-pPGH1 hybrid plasmid, also in *P. putida*, localized the chromosomal DNA between tandem IS21 sequences (Herrmann et al., 1988). Using a purified IS21 probe Nayundo et al. indicated that the DNA inserts of R68.45 R-primes in *Rhizobium* were inserted between the two IS21 sequences (Nayundo et al., 1987).

On the other hand, it has been proposed that the IS21 sequence is not involved in the formation of R-primes. Several lines of evidence support this claim. Beeching et al., demonstrated that during mobilization of R68.45 to *P. putida*, a copy of the IS21 sequence was lost (Beeching et

al., 1983). The plasmid containing a single IS21 sequence (pUU1) was subsequently used to form R-primes (Beeching et al., 1983). These experiments demonstrate that R-prime formation is not topographically linked to $(IS21)_2$ and could proceed without the involvement of $(IS21)_2$ (Beeching et al., 1983). In a similar experiment, Shah et al. used a derivative of R68.45 which contains a single IS21 (pJB3J1) to form R-primes in *B. japonicum* confirming that the $(IS21)_2$ is not involved in R-prime formation (Shah et al., 1989). Physical analysis of R-primes isolated from a single mating event in *Rhizobium* indicated that the insert is not located between the $(IS21)_2$ sequence and may vary in its locale (Johnston et al., 1978). Finally, analysis of R-primes formed in *Rhizobium* showed: i) the simultaneous loss of carbenicillin resistance and the auxotrophic phenotype and ii) the simultaneous transfer of carbenicillin resistance and the auxotrophic phenotype (Kowalczuk et al., 1981). These results indicate a linkage between the insert and the carbenicillin resistance gene (Kowalczuk et al., 1981). Examination of a physical map of R68.45 reveals that the kanamycin resistance gene, not the carbenicillin resistance gene, is located near the $(IS21)_2$ sequence. If the insert were localized between the $(IS21)_2$ sequence a loss of auxotrophy associated with the presence of an R-prime and

the simultaneous transfer of the prototrophic phenotype would be expected to be associated with the acquisition of kanamycin resistance. However, kanamycin resistance was not inherited, thus suggesting that the $(IS21)_2$ sequence may not be involved in the formation of R-primes.

One cannot discount the possibility that more than one mechanism may be involved in the formation of R-primes. Indeed, it has been shown that the mechanism may differ between *B. japonicum* and *R. fredii* (Shah et al., 1989). Also, the mechanism of R-prime formation may have nothing to do with a plasmid determined phenotype. The plasmid may simply act as suitable vector for transposable elements which leave the chromosome (Beeching et al., 1983).

What occurs after the association of R68.45 with the target DNA is even less understood than the mechanism involved in the initial interaction. After interaction R-prime formation might proceed in a similar manner to the formation of F-primes.

Isolation and Characterization of *P. putida* R-primes

Despite the many attributes of R68.45, it is a notoriously difficult plasmid with which to work. The difficulties associated with the plasmid are in part responsible for the gaps which remain in the understanding

of the basic mechanisms behind chromosome mobilization and R-prime formation. Advances have been made during this Masters project to overcome some of these obstacles. These advances include: the ability to isolate consistently the plasmid, and the introduction of pulsed field gel electrophoresis for the physical analysis of the plasmid.

The ability of R68.45 to form R-primes is well noted. In this study, interspecific matings were used to generate R-primes containing the *P. putida* methionine determinant. Three R-primes isolated from independent matings were transferred to *E. coli* and subsequently analyzed genetically and physically. The results of these analyses indicated that the R-primes were unstable in their basic genetic organization as indicated by the loss of the ability to confer tetracycline resistance to the host, and the inability of the plasmids to cotransfer relevant plasmid and chromosomal markers. The instability of R-primes can limit the usefulness of the plasmids as *in vivo* cloning vectors.

MATERIALS AND METHODS

1) Bacteria and culture conditions

The bacteria used during the experimentation are listed in table 1. Bacteria which contain R68.45 were maintained and grown in the presence of the antibiotics: kanamycin (300 μg/ml) and carbenicillin (500 μg/ml). The carbon source used to maintain and grow the bacteria varied among the organisms: *P. putidà* PRS 2003 containing R68.45 (provided by Dr. Mark Shanley, University of North Texas who originally obtained the culture from B. Holloway [Haas and Holloway, 1976]) was maintained on either defined minimal medium (Gerhardt et al., 1981) containing 0.3% glucose as a carbon source or Luria broth (LB) plates. Both media were supplemented with antibiotics. The methionine auxotrophs *P. aeruginosa* 27853-1s and 27853-11s (provided by Ms. Ping Wang) were maintained on minimal medium containing 0.3% glucose supplemented with methionine (0.3 mM final concentration) (Davis et al., 1980). Transconjugants containing R68.45 were maintained on 0.3% glucose plates supplemented with methionine and antibiotics. *P. aeruginosa* 27853-1s and 27853-11s transconjugants containing presumptive R68.45-*met* R-primes were maintained on 0.3% glucose plates supplemented with antibiotics. *E. coli* B and *E. coli* HB101 were maintained on LB plates. Transconjugants

of these were maintained on LB plates supplemented with antibiotics. Glycerol stocks of these cultures were maintained at -80° C in the same carbon source as used in the plate cultures with the exception of the *P. aeruginosa* 27853-1s and 27853-11s transconjugants containing the presumptive R-primes. These organisms were maintained in benzoic acid. Glycerol stocks of organism containing R68.45 were maintained in the presence of antibiotics. Liquid cultures of the organisms were grown to a cell density of approximately 2.45×10^8 cfu/ml at either room temperature (*P. putida* PRS 2003) or at 37° C (*P. aeruginosa* 27853-1s, *P. aeruginosa* 27553-11s, *E. coli* B, and *E. coli* HB101) in the appropriate carbon source and in the presence of antibiotics where required.

2) Plasmid isolation

a) To screen cultures for the presence of either R68.45 or R68.45 R-primes, small scale preparations of plasmid DNA were prepared according to the lysis by alkali method described by Sambrook et al. (1989) with slight modifications. One and one-half milliliters of overnight culture were pelleted by centrifugation (10,000 rpm [revolutions per minute], 5 minutes) and resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0) and 200 µl of solution II (0.2 N NaOH, 1%

SDS). Following the addition of solution II, the DNA solution was incubated for 5 minutes at room temperature. Following incubation, 150 μl of solution III (5 M potassium acetate, glacial acetic acid, water) were added and the mixture was placed on ice for 10 minutes. Following incubation on ice, the DNA solution was centrifuged (10,000 rpm, 10 minutes) to remove the cell debris. No phenol-chloroform extractions were performed. The DNA was precipitated with 2 volumes of ethanol on ice followed by centrifugation at 10,000 rpm for 25 minutes. The nucleic acids were dissolved in 20 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

b) Large-scale plasmid preparations for restriction analysis were prepared by alkaline denaturization. Cells were lysed, and the lysate was sheared, alkaline denatured, neutralized and extracted with phenol according to the procedure described by Currier and Nester (1976) with several modifications. A single colony was inoculated into one liter of LB and grown overnight to an OD_{650}-nm (optical density) of approximately 0.65 - 0.70 which corresponds to approximately 100 klett units (figure 2). The cells were pelleted by centrifugation (8000 rpm, 15 minutes) followed by washing in 10 ml of TE (0.05 M Tris-HCl and 0.005 M EDTA, pH 8.0) (Currier and Morgan, 1981).

Following washing, the bacteria were lysed by resuspending the cell pellet in a volume of TE plus 1% SDS and 500 μg/ml protease (pronase E) equal to two times the klett value. After incubation at 37° C for at least one hour, a clear lysate was formed. Shearing to reduce the viscosity of the lysate was accomplished by mixing on a magnetic stirring pad on low speed (100 - 150 rpm) for 5 minutes. Alkaline denaturization of the sheared lysate was performed by adjusting the pH to 12.1 - 12.3 using 3 N NaOH with continuous stirring. After stirring for 10 minutes, the lysate was neutralized by adjusting the pH to 8.5 - 9.0 using 2.0 M Tris-base [tris(hydroxylmethyl)aminomethane] pH 7.0. The lysate was maintained at this pH for 5 minutes with continuous stirring. Following neutralization the lysate was adjusted to 3% (w/v) NaCl by the addition of solid NaCl. To remove denatured proteins, an equal volume of phenol equilibrated in 3% NaCl was added to the lysate and stirred for 5 minutes, followed by centrifugation (3000 rpm, 12 minutes) to separate the phases. Chloroform extraction was omitted. DNA, present in the aqueous phase, was precipitated by the addition of 2 volumes of cold (-20°C) ethanol in the presence of 1/30 volume of 3 M sodium acetate (pH 5.0) (Perbal, 1988). The DNA was allowed to precipitate at least 24 hrs at -20° C; the precipitate was collected by centification and redissolved in 10 ml TE (10

mM Tris-HCl, 1 mM EDTA, pH 8.0).

Covalently closed circular DNA was isolated by cesium chloride-ethidium bromide density gradients. The DNA solution, in 13 ml of TE buffer, was added to 13 g of cesium chloride, and to this solution 1.0 ml of ethidium bromide (10 mg/ml, in TE) was added. The solution was centrifuged to equilibrium using a Beckman Type 70.1 Ti rotor at 36,000 rpm, 48 hrs, 25° C. Ethidium bromide was removed by extraction with butanol saturated with TE. Cesium chloride was removed by dilution and ethanol precipitation (Davis et al., 1980). The DNA was precipitated by centrifugation (8,000 rpm, 10 minutes) followed by washing with 5 ml 70% ethanol. Nucleic acids were resuspended in 1.0 ml TE and stored in 100 μl aliquots at -20° C.

3) Restriction endonuclease digests and agarose gel electrophoresis

a) Electrophoresis of plasmid DNA isolated from the small scale preparations was performed as follows. Twenty microliters of DNA solution (approximately 1 - 2 μg DNA/μl) was electrophoretically separated on a 3 mm, 1% agarose gel prepared in 0.5x TBE (0.045 M boric acid, 0.045 M Tris-base, 0.001 M EDTA) for 2.5 hrs at 100 V. The running buffer was the same as that used to prepare the agarose. Lambda *Hind*III digests were used as molecular weight markers. DNA was stained with ethidium bromide (0.5 μg/ml) and visualized

using a 260-nm ultraviolet transilluminator.

b) Restriction digestion of R68.45 and pulsed field gel electrophoresis of the digested DNA was performed as follows. The following restriction enzymes were purchased from New England Biolabs: *Hind*III, *Sal*I, and *Eco*RI. Reaction mixtures contained 20 μl of cesium chloride purified DNA (0.20 - 0.30 μg DNA/μl) in TE buffer, 3 μl of 10x reaction buffer, and 1 μl of restriction enzyme (20 units). In reactions containing *Sal*I the reaction mixture was supplemented with 100 μg/ml bovine serum albumin. For simultaneous digestion with two restriction endonucleases, the 10x buffer which produced optimal activity of both enzymes was used: *Sal*I buffer for digests including *Sal*I in combination with *Hind*III or *Eco*RI, and *Eco*RI buffer for *Eco*RI in combination with *Hind*III. The enzyme in limiting conditions (*ie.*, not in optimal buffer conditions) was added at a higher concentration (2 μl/reaction mixture). The reactions were incubated at 37° C for 4 hrs, at which time the reaction was stopped by placing the reaction mixture at 65° C for 10 minutes. Twenty microliters of sample were then subject to pulsed field gel electrophoresis using the Biorad CHEF-DRII (contour-clamped homogeneous electric field) electrophoresis apparatus. The sample was electrophoresed through a 13 cm gel of 5 mm thickness consisting of 1.0% agarose, made in 0.5x TBE, for 18 hrs at

10 V/cm with a 1 -10 second ramp time. Lambda *Hind*III DNA and a 5 kb ladder, purchased from Biorad, were used as size standards. The electrophoresis buffer used was 0.5x TBE. Following electrophoresis the gels were stained with ethidium bromide (0.5 μg/ml) for 30 minutes and then destained in water for 30 minutes. Bands were visualized with 260-nm ultraviolet transilluminator.

4) Plasmid elimination

R68.45 was cured from *P. putida* PRS 2003 by treatment of the cells with ethidium bromide (Kowalczuk et al., 1981). Overnight cultures were diluted to 1.26 x 10^7 cfu/ml and supplemented with 100 μg/ml ethidium bromide. The culture was then grown to a cell concentration of 1.23 x 10^8 cfu/ml. Dilutions of the culture were plated onto LB and incubated at 30° C overnight. Colonies from the LB plates were replica plated by stippling onto glucose plates and glucose plates supplemented with kanamycin (300 μg/ml). Colonies which grew on the glucose but not on the glucose-kanamycin plates were screened for the presence of the plasmid by small scale plasmid preparations.

5) Determination of the antibiotic sensitivity

To define the selective conditions used during bacterial matings and to confirm the presence of R68.45 in *P. putida* 2003, the antibiotic profiles of the bacteria were

generated using penicylinders (Fisher). One hundred microliters of overnight culture of the test strain grown in LB were plated onto LB plates. Following plating, 150 μl of various concentrations of antibiotic were inoculated into each cylinder, which had been previously placed on the lawn of bacteria. After incubation at 30° C overnight, the sensitivity was determined by measuring the area of inhibition surrounding the penicylinder. The antibiotic profile of *P. aeruginosa* 27853 had been determined previously (D. Dutt, personal communication).

6) Determination of carbon source utilization and confirmation of auxotrophy

Identification of carbon sources which are selective for or against an organism and the confirmation of an auxotrophic phenotype were determined as follows. Overnight cultures of bacteria were washed 3 times in a minimal salts solution (0.02 M KH_2PO_4, 0.01 M NH_4Cl, 0.02 M Na_2HPO_4) and then resuspended in 1 ml of minimal salts for dilution and plating. Dilutions of bacteria were plated onto the test carbon source and incubated for at least 3 days while checking for growth at 24 hr intervals. The methionine auxotrophy of *P. aeruginosa* 27853-1s and 27853-11s was confirmed by plating the organism onto glucose plates which either lacked or contained methionine. The inability of *P.*

putida to utilize benzoic acid was confirmed by plating this organism onto plates which contained benzoic acid as the sole carbon source and checking for the absence of growth.

7) Species identification

The species of the bacteria was confirmed using Biolog GN Microplates. Biolog plates are 96 well microplates designed to test the ability of a microorganism to oxidize a selection of 95 carbon sources. Utilization of a carbon source is indicated by the production of a purple dye due to the reduction of a colorless tetrazolium dye to a purple formazan product. The latter produces a pattern which constitutes a "metabolic fingerprint" of the inoculated organism. Twenty-five milliliters of a 2.45 x 10^8 cfu/ml culture in TSB (tryptic soy broth) were pelleted by centrifugation at 8,000 rpm for 10 minutes. Following centrifugation the cells were washed 3 times with 10 ml minimal salts solution. Following washing, 20 ml of 0.85% saline solution was adjusted to an OD_{590}-nm of 0.5. Each well of the microtiter plate was inoculated with 150 μl of culture, followed by incubation at 30° C overnight. Color formation was determined by eye (purple = positive reaction; clear = negative reaction). Species identification was determined using the microlog data base. Species of *E. coli* are not efficiently identified using Biolog GN plates

(Biolog manual); therefore the species identification for *E. coli* B was confirmed by streaking the organism onto EMB plates (Levine, 1918).

8) Bacterial matings and selective conditions

a) *P. Putida* PRS 2003 x *E. coli* HB101: This mating was performed to determine if the antibiotic resistant phenotype associated with R68.45 was capable of being expresssed in *E. coli*. A modified liquid mating technique described by Chatterjee (1980) was used to transfer the plasmid. Exponential cultures (2.45×10^8 cfu/ml) of donor in LB supplemented with antibiotics and recipient in LB were pelleted by centrifugation and washed with minimal salts solution, then resuspended in 1 ml of minimal salts solution. From the cell suspension 5 ml of LB was adjusted to a cell concentration of 1.23×10^8 cfu/ml. Donor and recipients were mixed at a 1:1 ratio and incubated without shaking for 16 hrs at 30° C. The mating mixture was then plated onto selective medium (figure 3). *E. coli* transconjugants containing R68.45 were selected by plating the mating mixture onto a selective medium containing LB supplemented with kanamycin ([25 μg/ml] to select against the recipient) which was incubated at 42° C (to select against the donor). Colonies which grew under the selective conditions were then replica plated by stippling onto LB

plates supplemented with kanamycin (300 μg/ml), carbenicillin (300 μg/ml), and tetracycline (100 μg/ml). The frequency of plasmid transfer was calculated per number of donor cells.

b) *P. putida* PRS 2003 x *P. aeruginosa* 27853-1s and 27853-11s: R68.45 R-primes containing a methionine determinant capable of complementing the methionine auxotrophy of *P. aeruginosa* 27853-1s and 27853-11s were generated by interspecific matings between *P. putida* PRS 2003 and *P. aeruginosa* 27853-1s and 27853-11s. The plasmid was transferred using a modified membrane mating technique described by Beeching et al. (1983). The donor strain, *P. putida* 2003 was grown in LB supplemented with antibiotics. The recipient strain, *P. aeruginosa* 27853-1s or 27853-11s was grown in LB. Exponential phase cultures of donor and recipient organisms (2.45 x 10^8 cfu/ml) were centrifuged (8,000 rpm, 10 minute) and resuspended in 5.0 ml of minimal salts solution. The suspensions were mixed and vacuum filtered through a 0.45 μm pore size membrane filter. The filter was transferred to an LB plate and incubated at 30° C for 16 hrs. Following incubation, the membrane was transferred to a sterile test tube containing 2.5 ml of minimal salts solution. Samples of mating mixture were then plated onto selective media (figure 4). *P. aeruginosa*

transconjugants containing R68.45 were selected by plating onto a selective medium containing benzoic acid (to select against the donor), carbenicillin ([500 μg/ml] to select against the recipient), kanamycin ([300 μg/ml] to maintain plasmid organization) (Currier and Morgan, 1982), and methionine (to allow the auxotrophic transconjugant to grow). The selective plates were subsequently incubated at 42° C for additional selection against the donor. Transconjugants containing R68.45-*met* R-primes were selected using the same conditions except that methionine was omitted from the selective medium. Colonies which grew on the selective plates were replica plated by stippling onto glucose plates supplemented with kanamycin (300 μg/ml) and carbenicillin (300 μg/ml). Frequency of transfer and R-prime formation was calculated per number of donor cells.

c) *P. aeruginosa* 27853-1s or 11s transconjugants containing R68.45-*met* x *E. coli* B: To isolate the R-primes for further analysis, it was necessary to mobilize them to *E. coli* B. Membrane filter matings, as previously described, were used to transfer the plasmid. The donor organisms, *P aeruginosa* 27853-1s or 27853-11s containing R68.45-*met* were grown to exponential phase (2.45×10^8 cfu/ml) in benzoic acid supplemented with kanamycin (300 μg/ml) and carbenicillin (500 μg/ml). The recipient *E. coli*

B was grown to exponential phase in LB. Selection for transconjugants was based on the ability of the recipient to grow anaerobically, while the donor cannot (figure 5). The mating mixture was plated onto LB plates supplemented with kanamycin and carbenicillin ([200 μg/ml] to select against the recipient), and incubated in an anaerobic chamber for 24 hrs at 32° C. Colonies which grew under these conditions were subsequently replica plated by stippling onto LB plates supplemented with kanamycin and carbenicillin (200 μg/ml). Frequency of transfer was calculated per number of donor cells.

d) *E. coli* B transconjugants containing R68.45-*met* x *P. aeruginosa* 27853-1s or *P. aeruginosa* 27853-11s. A feature of R-primes is the ability to cotransfer chromosomal markers and plasmid markers (Holloway, 1978). Cotransfer of markers was examined by mating the *E. coli* B transconjugants and the original methionine auxotrophs (figure 6). Plasmid transfer was accomplished using filter matings. The frequency of cotransfer of the chromosomal and plasmid markers was determined by selection on itaconic acid (selects against *E. coli* B) - carbenicillin ([500 μg/ml] (selects against the recipient) plates. The frequency of plasmid marker transfer was determined by selection on itaconic acid-methionine-carbenicillin plates.

9) Determination of R-prime stability

Prime plasmids are notoriously unstable. The method described by Chatterjee (1980) was used to determine the stability of the R-primes. Purified clones of *P. aeruginosa* transconjugants containing the R-primes were grown in benzoic acid supplemented with carbenicillin (500 μg/ml) and kanamycin (300 μg/ml). The culture was then plated onto LB plates. Cells from the LB plates were inoculated into fresh LB broth to an initial density of 5×10^5 cfu/ml. Cultures were grown for 16 hrs at room temperature and serially diluted; samples of appropriate dilutions were plated onto LB plates to isolate single colonies. Individual colonies (50 from each plate) were replica plated by stippling onto LB plates and allowed to grow for 16 hrs. The resulting colonies were subsequently replica plated onto glucose medium; glucose medium supplemented with kanamycin (300 μg/ml) and carbenicillin (500 μg/ml) and glucose medium supplemented with kanamycin (300 μg/ml), carbenicillin (500 μg/ml), and methionine. Clones growing in the absence of methionine were scored after 16 hrs of incubation.

RESULTS

Confirmation of the presence of R68.45 in *P. putida* PRS 2003

Prior to the generation of the R-primes, it was essential to confirm that the donor, *P. putida*, contained the correct plasmid (*ie.*, R68.45). There are two main criteria which are used to confirm the presence of a plasmid: 1) a demonstration of its presence through physical characterization, and 2) genetic evidence. In this study, both forms of evidence were provided to confirm the presence of R68.45 in the donor species.

1. Isolation and restriction characterization of the plasmid in *P. putida* PRS 2003

The presence of a plasmid in *P. putida* PRS 2003 was demonstrated by plasmid preparations (figure 7, lane 2). Conventional electrophoresis is unable to resolve accurately DNA molecules above 20 kb in size (Cantor, 1988). For this reason, pulsed field gel electrophoresis was used for the molecular weight determination and restriction analysis of R68.45 (figure 8). Covalently closed circular DNA molecules are unable to enter into pulsed field gels (Smith and Cantor, 1987); therefore, to determine the molecular weight of a plasmid it is necessary to convert it to a linear form. The restriction map of R68.45 (figure 1) indicates that R68.45 contains unique restriction sites for *Hind*III and

*Eco*RI. Restriction of the plasmid isolated from *P. putida* 2003 with *Hind*III and *Eco*RI produced one band of approximately 60 kb in size (figure 8, lanes 4 and 5). This measurement is in agreement with previously published size estimates of 37.4 Mdal or 58.9 kb (Jacob et al., 1977). Restriction of the plasmid with *Sal*I produced two bands of approximately 40 kb and 19 kb (figure 8, lane 6), which is in agreement with previously published results for the restriction of R68.45 with SalI (Currier and Morgan, 1981). Double digestion of the plasmid with various combinations of *Hind*III, *Eco*RI and *Sal*I produced the following size fragments: *Sal*I and *Hind*III produced three bands of approximately 30 kb, 19 kb and 6.5 kb (figure 8, lane 7); *Eco*RI and *Sal*I produced three bands of approximately 25 kb, 19 kb and 14 kb (figure 8, lane 8); *Eco*RI and *Hind*III produced two bands of approximately 40 kb and 20 kb (figure 8, lane 9). The summation of the fragments produced in the double digest is roughly 60 kb. The number of fragments produced by the double digests is in agreement with the restriction map of R68.45 (figure 1). These results strongly suggest that the plasmid isolated from *P. putida* PRS 2003 is R68.45.

2. Genetic evidence for the presence of R68.45 in *P. putida* PRS 2003

a. Plasmid curing

R68.45 codes for resistance to several antibiotics, namely, kanamycin, carbenicillin, and tetracycline (Haas and Holloway, 1976). The loss of these phenotypes associated with the loss of the plasmid from *P. putida* PRS 2003 would imply that the plasmid present in *P. putida* PRS 2003 is R68.45. The plasmid was cured from *P. putida* PRS 2003 by ethidium bromide treatment (Kowalczuk et al., 1981). Two hundred colonies from the ethidium bromide treatment were replica plated by stippling onto glucose plates and glucose plates supplemented with kanamycin. One colony designated *P. putida* PRS 2003b_2 was able to grow on the glucose plates but was unable to grow on the glucose plates supplemented with kanamycin (300 μg/ml). This would indicate that *P. putida* 2003b_2 no longer expresses kanamycin resistance and has possibly lost the plasmid. To confirm that *P. putida* PRS 2003b_2 no longer contained a plasmid, plasmid preparations were performed on *P. putida* PRS 2003 and *P. putida* 2003b_2. The results shown in figure 9, lane 3 clearly show that *P. putida* PRS 2003b_2 lacks a plasmid. *P. putida* PRS 2003 and *P. putida* PRS 2003b_2 were subsequently plated onto LB plates supplemented with kanamycin (300

μg/ml), carbenicillin (300 μg/ml), and tetracycline (100 μg/ml). *P. putida* PRS 2003 was able to grow on these plates, however *P. putida* PRS 2003b_2 was not. The antibiotic profiles of the organisms are shown in figure 10. *P. putida* 2003b_2 has become sensitive to kanamycin at 25 μg/ml, carbenicillin at 1000 μg/ml, and tetracycline at 10 μg/ml upon loss of the plasmid. These results complement the physical evidence to suggest that the plasmid carried by *P. putida* PRS 2003 is R68.45.

b. Conjugal transfer of the plasmid to a new host

A property of conjugative plasmids is the ability to transfer phenotypes associated with the plasmid to a new bacterial host. Transfer of the plasmid was accomplished by mating *P. putida* 2003 and *E. coli* HB101 (figure 3). A prerequisite for bacterial matings is the ability to select for the transconjugants and against the donor and recipient. Selection may be based on several criteria including carbon source utilization, permissible temperature, availability of oxygen, dependence on growth factors and antibiotic sensitivity. Prior to the mating, the minimal inhibition concentrations (MICs) for *E. coli* HB101 to various antibiotics was determined. In particular, MICs for kanamycin, carbenicillin and tetracycline were determined to be 10 μg/ml for kanamycin and tetracycline, and 25 μg/ml for

carbenicillin. Prior to the mating, several control experiments were performed. First, it was affirmed that the donor was unable to grow at 42° C, and able to grow in the presence of antibiotics. Second, it was verified that the recipient was able to grow at 42° C, but could not grow in the presence of kanamycin (25 μg/ml). Third, the species identification of the donor was verified as *P. putida* using Biolog plates. A representative Biolog printout is shown in figure 11, and Biolog results in table 2. Biolog analysis was unable to confirm the identity of the recipient as *E. coli*. However, a metabolic pattern was generated for comparison to the transconjugant. Finally, the presence of a plasmid in the donor and the absence of a plasmid in the recipient was verified (figure 12). R68.45 was transferred to HB101 at a frequency of 1.31×10^{-2} (table 3). The presence of a plasmid in the transconjugants was revealed through plasmid isolation (figure 13). Biolog analysis of a transconjugant generated a pattern similar to that of the recipient (data not shown) implying that the transconjugant is *E. coli* HB101. The antibiotic profile of an HB101 transconjugant (figure 14) illustrates that as a consequence of acquiring the plasmid from *P. putida* 2003 the transconjugant became resistant to kanamycin, carbenicillin and tetracycline. The conjugation results further indicated

that the plasmid contained in *P. putida* 2003 is R68.45.

Generation and Isolation of R68.45-*met* R-primes

1. Control experiments prior to mating

The inability of the donor to grow on both benzoic acid and the selective medium (figure 4), and its inability to grow at 42° C were confirmed by plating the donor onto benzoic acid plates, LB plates, and benzoic acid-kanamycin-carbenicillin plates incubated at 42° C. The LB and benzoic acid plates were also incubated at 30° C. Growth was observed only on the LB plates incubated at 30° C, confirming the phenotype of the donor. Similar experiments were performed to verify the methionine auxotrophy of *P. aeruginosa* 27853-1s and 27853-11s; their ability to utilize benzoic acid and to grow at 42° C; and their sensitivity to carbenicillin. The recipients were plated onto glucose plates; glucose-methionine plates; benzoic acid plates; benzoic acid-methionine plates; benzoic acid-methionine-kanamycin-carbenicillin plates; and benzoic acid kanamycin-carbenicillin plates. All plates were incubated at 42° C. Growth was observed only on the glucose-methionine and benzoic acid-methionine plates The results confirm the phenotypes of the recipient and the appropriateness of selective conditions used during the mating. The presence of R68.45 in the donor and the absence of a plasmid in the recipients were confirmed by plasmid isolation (figure 15).

The final control experiment was to affirm the species of the donor and recipients prior to mating. The results shown in table 2 verify the donor as *P. putida* and the recipients as *P. aeruginosa*.

2. Matings

Putative R68.45-*met* R-primes with the ability to complement the methionine auxotrophy of *P. aeruginosa* 27853-1s and *P. aeruginosa* 27853-11s were generated by interspecific matings between *P. putida* 2003 and the *P. aeruginosa* recipient. Three independent matings as diagramed in figure 4 were conducted: two between *P. putida* 2003 and *P. aeruginosa* 27853-1s and one between *P. putida* 2003 and *P. aeruginosa* 27853-11s. R68.45 was transferred at a frequency of 10^{-1} in all matings (table 4). The frequency of R-prime formation varied: 9.9×10^{-7} for the first mating between 2003 and 27853-1s; 1.7×10^{-4} for the second between 2003 and 27853-1s, and; 1.4×10^{-6} for the mating between 2003 and 27853-11s (table 4).

3. Evidence for the presence of plasmids in the *P. aeruginosa* transconjugants and the presence of R-*met* R-primes

P. aeruginosa transconjugant selected in the presence of methionine demonstrated the presence of a plasmid which migrated to a similar position as R68.45 through

conventional gel electrophoresis (figure 16). Physical examination of the transconjugants containing putative R-primes (figures 17, 18, and 19) displayed the presence of a plasmid which migrated slightly higher then R68.45. The ability of the *P. aeruginosa* transconjugants containing putative R-primes to grow in the absence of methionine (*ie.*, the conversion to prototrophy) and in the presence of antibiotics provided genetic evidence for the presence of R68.45-*met* R-primes. The species of the transconjugants was identified as *P. aeruginosa* using Biolog plates (table 2). Three R-primes, one isolated from each mating, were chosen for further study. These were designated *P. aeruginosa* 27853-1s pPM21; *P. aeruginosa* 27853-1s pPM15, and *P. aeruginosa* 27853-11s pPM4.

4. Stability of the R-primes

One feature of prime plasmids is instability (Johnston et al., 1978). The R-*met* containing strains were grown in the absence of methionine selection for several generations. After growth in the non-selective conditions, fifty colonies from each transconjugant were tested for methionine prototrophy and resistance to carbenicillin. All colonies tested remained methionine prototrophs and resistant to carbenicillin signifying that the R68.45-*met* primes are stable in the *P. aeruginosa* host for at least the number of generations tested.

Movement of the R-primes into *E. coli* B

The production of alginate by *P. aeruginosa* necessitated the movement of the R68.45-*met* primes from *P. aeurginosa* to *E. coli* B. The ability of *P. aeruginosa* transconjugants to grow on a wide range of carbon sources including maltose, lactose, D-galactose, sucrose and D-mannitol, originally believed to be selective against them (Kreig, 1984), compelled the use of an alternative selective regime. *Pseudomonas* is phenotypically an obligate aerobe and *E. coli* a facultative anaerobe (Kreig, 1984). These phenotypes were confirmed by incubation in an anaerobic chamber. The inhibitory concentration of carbenicillin and kanamycin to *E. coli* B was determined to be 100 μg/ml. Based on the above results, selection was based on growth under anaerobic conditions in the presence of kanamycin and carbenicillin both at a final concentration of 200 μg/ml (figure 5). The presence of the plasmid in the donor and the absence of a plasmid in the recipient was confirmed through plasmid preparations (data not shown). pPM15 transferred at a frequency of 7.0×10^{-4}, pPM21 at a frequency of 6.2×10^{-4}; and pPM4 at a frequency of 3.9×10^{-4} (table 5). The appearance of dark red colonies producing a metallic green sheen on EMB plates verified the transconjugants as *E. coli*. Figures 20, 21, and 22

illustrates the acquisition of a plasmid by the *E. coli* B transconjugants which is comparable in size to the plasmid in the donor.

1. Ability of the plasmids in *E. coli* B to express plasmid markers

The *E. coli* B transconjugants containing the R-*met* primes were maintained on LB-kanamycin-carbenicillin. Plasmids isolated from the *E. coli* B transconjugants stored under these conditions no longer migrated to a position similar to that of the original R-prime, but to a lower position (figure 23: lanes 2, 4, and 6 are *P. aeruginosa* transconjugants: lanes 3, 5, and 7 are the *E. coli* B transconjugants). Streaking the *E. coli* transconjugants onto LB-kanamycin (300 μg/ml) plates , LB-carbenicillin (300 μg/ml) and LB-tetracycline (50 μg/ml) indicated that the *E. coli* B transconjugants no longer expressed the tetracycline marker, whereas the original *P. aeruginosa* host expressed all antibiotic markers. These results may suggest that the plasmid is unstable or that the tetracycline determinant is unable to be expressed in the *E. coli* host.

2. Ability of the plasmid in *E. coli* B transconjugants to cotransfer plasmid markers and the methionine determinant to *P. aeruginosa* 27853-1s or 27853-11s

A feature of prime plasmids is the ability to cotransfer plasmid and chromosomal markers. To test this,

E. coli B transconjugants containing the primes were mated with either *P. aeruginosa* 27853-1s or 27853-11s depending on which organism was used to originally generate the R-prime. The mating strategy and selection is shown in figure 6. Similar controls to the ones for previous matings were performed. The inability of the donor to grow on itaconic acid was confirmed prior to the mating, as was the methionine auxotrophy of the recipient and ability of the recipient to utilize itaconic acid in the absence of antibiotics (data not shown). Also, the presence of the plasmid in the donor and the absence of a plasmid in the recipients was confirmed by plasmid isolation (data not shown). The frequency of plasmid marker transfer was 1.6×10^{-3} for pPM4, 2.3×10^{-4} for pPM15, and 7.0×10^{-5} for pPM21 (table 6). The ability of the plasmid to transfer the methionine determinant was much lower: 1.1×10^{-5} for pPM4, 6.6×10^{-7} for pPM15, and 2.3×10^{-7} for pPM21 (table 6). Both the carbenicillin resistant transconjugants and the methionine prototrophs derived from the mating revealed the presence of a plasmid the same size as the plasmid present in the donor, *E. coli* B (results not shown). Streaking the suspect transconjugants onto EMB plates produced pink colonies. This observaton would indicate that the transconjugants are not *E. coli* and are more likely *P.*

aeruginosa. The results of this experimentation indicate that the plasmids contained in the *E. coli* B transconjugants are able to transfer the methionine marker and carbenicillin resistance, but marker transfer is not at equivalent rates. The inability to cotransfer the markers at equivalent rates is most likely a reflection of prime plasmid instability.

3. Restriction analysis of the plasmids isolated from the *E. coli* B transconjugants

Plasmids isolated from the *E. coli* B transconjugants were restricted with *Eco*RI and double digested with *Hind*III and *Sal*I. R68.45 isolated from *P. putida* 2003 was restricted in the same manner. Digests of each plasmid and a mixture of the plasmids restricted with the same enzyme were electrophoresed through pulsed field gel electrophoresis to determine if any differences were apparent between R68.45 and the plasmid isolated from the *E. coli* B transconjugant. Figure 24 shows a representative digestion pattern from such an experiment. Lane 4 and 5 respectively are R68.45 and pPM21 restricted with *Eco*RI, lane 6 is the mixture of the two plasmids restricted with *Eco*RI. pPM21 appears to be migrating slightly lower then R68.45. Lanes 7 and 8 respectively are pPM21 and R68.45 restricted with *Hind*III and *Sal*I, lane 9 is the mixture of the two plasmids. The restriction of R68.45 produces a 30 kb, a 19kb and a 6.5 kb band (lane 8). The restriction of

pPM21 produces the 30 kb and 19 kb bands but the 6.5 kb band is absent (lane 7). however, a band of less then 5.0 kb is evident in pPM21 that is absent in R68.45. These results indicated that pPM21, and the other plasmids in the *E. coli* B transconjugants had lost a DNA segment in the 6.5 kb *Hind*III and *Sal*I fragment.

DISCUSSION

Initially, one might think that broad-host-range plasmids, including R68.45, would serve as the center of prokaryotic molecular biology due to their ability to transfer to almost any gram-negative host. However, the use of this plasmid is not a common practice. The main reason for the infrequent use is the difficulty in adapting basic molecular biology technology to its use. In particular, the large size of plasmid and plasmid instability hinder its isolation and characterization.

The present work is characterized by several achievements which advance the potential use of this plasmid. One achievement has been the reliable isolation of purified R68.45 in yields sufficient for physical characterization of the plasmid and constituent fragments. Also, the restriction characterization of the plasmid has been enhanced by the introduction of pulsed field gel electrophoresis.

Versatile selective regimes have been developed for interspecific as well as intergeneric crosses. These crosses were utilized to generate R68.45-*met* primes for examination. The examination of the R-primes involved the correlation between genetic properties and physical characterization of plasmid transfer. Finally, quantitative

data on the transmission of R68.45, the formation of R-primes, and the cotransfer of relevant genetic markers was generated.

The results of this experimentation have given rise to important questions concerning the traditional way of thinking about plasmid structure and the extent to which this structure may be altered. Also, implications have been made about the usefulness of R68.45 primes as *in vivo* cloning vectors.

Confirmation of the presence of R68.45 in *P. putida* 2003 through physical and genetic characterization

Verification of the presence of R68.45 in *P. putida* was achieved by physical and genetic criteria. Physical analysis of the plasmid was accomplished through restriction analysis using pulsed field gel electrophoresis (figure 8). Previous restriction characterization of IncP-1 plasmids had been conducted using conventional electrophoresis (Currier and Morgan, 1981). Conventional electrophoresis limits the accurate resolution of DNA fragment to those fragments which are less then 20 kb is size. Pulsed field gel electrophoresis extends the resolving power of electrophoresis to include the reliable resolution of megabase sized fragments. Through pulsed field gel electrophoresis the molecular weight of the plasmid was

confirmed to be approximately 60 kb, in agreement with previously published estimates (Jacob et al., 1977). Restriction of the plasmid with the enzyme *Sal*I generated a restriction pattern that was consistent with and, thus, substantiated the pattern previously published by Currier and Morgan 1981. Finally, restriction with *Sal*I in combination with *Hind*III or *Eco*RI and restriction with *Eco*RI in combination with *Hind*III generated the number of fragments expected based on the restriction map of R68.45 (figure 1). The physical data provide evidence that the plasmid contained in *P. putida* 2003 is R68.45.

Plasmids have been isolated and characterized which do not have any genetic function ascribed to them (Stanish, 1988). For this reason physical characterization of a plasmid, although it is essential to confirm its presence, cannot be divorced from genetic analysis. Two forms of genetic standards, plasmid curing and conjugal transfer of the plasmid, confirmed the presence of R68.45 in *P. putida* 2003.

The loss of bacterial properties encoded by a plasmid is often used as an indicator of the absence of a particular plasmid. Curing of the plasmid from *P. putida* 2003 to generate *P. putida* 2003b_2 rendered *P. putida* 2003b_2 sensitive to kanamycin, carbenicillin, and tetracycline. Resistance

to these particular antibiotics are encoded by R68.45. The acquired sensitivity to these antibiotic due to the loss of the plasmid would indicate that the plasmid present in *P. putida* to be R68.45.

The transfer of genetic properties from one bacterium to another is a second form of presumptive genetic evidence for the presence of a particular plasmid. The ability of the plasmid in *P. putida* to transfer tetracycline, kanamycin, and carbenicillin resistance to *E. coli* HB101, therefore, indicates that the plasmid contained in *P. putida* is R68.45.

Generation and isolation of R-primes

Interspecific matings involving R68.45 have been used to isolate R-primes carrying chromosomal genes from *P. aeruginosa* (Morgan, 1982), *P. putida* (Bray et al., 1987), and *R. leguminosarum* (Johnston et al., 1978b). In this study, the use of selection for the transfer of carbenicillin resistance and methionine prototophy in three independent matings involving R68.45 has been used to select for putative R68.45 R-primes which have transferred the *P. putida* methionine determinant to *P. aeruginosa*. Genetic evidence for the presence of the R-primes was provided by demonstrating the conversion of the recipients from methionine auxotrophs to prototrophs without the apparent

occurrence of reversion. Correlation between the genetic observations and the physical demonstration of the acquisition by the transconjugants of a plasmid slightly larger then R68.45 (figures 17, 18, 19) substantiated the presence of the R-*met* primes. The frequency of plasmid transfer was consistently 10^{-1} in all matings (table 4). Frequencies of R68.45 transfer of this magnitude have been previously noted (Banfalvi et al., 1983). The frequencies of R-prime formation varied from 10^{-4} to 10^{-7} (table 4). The frequencies of *P. putida* R-prime formation in *P. aeruginosa* noted here are slightly higher then those previously reported, 10^{-8} to 10^{-10} (Bray, 1987). The reason for the frequency of R-prime formation is unclear; however, Bray had previously noted that the detection of R-primes carrying different markers on the *P. putida* chromosome varied in their frequency. In this study, one R-prime from each mating, specifically, pPM4, pPM15, and pPM21, were used for subsequent experiments.

Examination of the R-prime stability

A feature of R-primes is instability in the absence of environmental conditions which require the expression of the chromosomal insert (Johnston et al., 1978b; Morgan, 1982; Banfalvi et al., 1983). Under non-selective conditions in

the *P. aeruginosa* host the R-primes generated in this study were stable. Several other investigators have also reported the isolation of stable R-primes (Chatterjee, 1980; Kiss et al., 1980).

Transfer of the R-primes to *E. coli* B

Through intergeneric matings the R-primes were transferred to *E. coli* B. Genetic and physical analysis of the plasmids in *E. coli* B yielded intriguing results. Following the transfer of pPM4, pPM15, and pPM21, the plasmids no longer expressed the tetracycline resistance marker. It cannot be stated explicitly whether the lack of tetracycline resistance in the *E. coli* B host is due to the physical loss of a plasmid segment or is a reflection of the genetic distance between *P. aeruginosa* and *E. coli*, a circumstance which may lead to the irregular expression of the marker. A way to distinquish between these two possiblities would be to perform a backcross between the *E. coli* B transconjugants and *P. aeruginosa*, followed by testing for the expression of the tetracycline resistance marker in *P. aeruginosa*. If the marker is expressed, this would imply that the plasmid had not be structurally altered, and that the tetracyline resistance marker is simply not expressed in *E. coli* B.

The plasmids contained in the *E. coli* hosts are assumed

to be methionine containing R-primes. One distinctive feature of R-primes is the ability to transfer relevant plasmid markers and chromosomal markers at similar frequencies. The plasmids contained in the *E. coli* hosts were able to transfer genes for methionine prototrophy and carbenicillin resistance; however, the transfer was not at equivalent or even similar frequencies (table 6). The inability of R-primes to cotransfer markers at the anticipated frequencies has been observed previously (Holloway, 1978; Morgan, 1982). Therefore, the possibility that the plasmid contained in the *E. coli* transconjugants is an R-prime cannot be completely discounted.

Isolation of pPM15, pPM21 and pPM4 from the *E. coli* transconjugants initially showed a plasmid equal in size to the plasmid contained in the *P. aeruginosa* donor (figures 20, 21, 22). However, subsequent isolation of plasmids obtained from cultures maintained in LB-Km-Cb revealed plasmids which were smaller then the plasmid in the *P. aeruginosa* donor (figure 23). It is interesting to note that the loss incurred by all three plasmids appears to be similar in size, despite the fact that these plasmid were isolated from independent matings. The plasmids were examined in more detail by restriction analysis (figure 24). This analysis revealed that the plasmids differed from R68.45 in the 6.5 kb *Hind*III/*Sal*I fragment. All three

plasmids no longer contained the 6.5 kb fragment but a smaller fragment. The exact size of this fragment was not precisely determined due to the lack of suitable size standards. It cannot be definitively stated that the loss of the DNA fragment is associated with either the loss of the tetracycline resistance or the lack of cotransfer of relevant markers since the location of the methionine marker was never clearly established and the time of DNA loss is unknown.

Some Interpretations

Taken collectively, the interpretation of the results obtained from the genetic and physical analysis of the plasmids in *E. coli* B is not a simple issue. The speculation that the plasmids in *E. coli* B remain R-primes despite the smaller size, may be supported if one remembers that to form an R-prime, R68.45 must initially interact with the chromosome and then be excised. If the excision is imprecise there should be no expectation that the resultant prime be larger. Also, the difference in size may be a reflection of plasmid instability in areas other then the chromosomal insert. Similar results have been reported previously (Morgan, 1982).

The loss of tetracycline resistance and the inability of the plasmids to cotransfer relevant marker most likely

reflects plasmid instability. Such loss gives rise to questions concerning the inherent structure of plasmids and the limit to which this structure may be changed without detrimental effects on plasmid stability. Bacterial chromosomes are believed to possess a defined structure, size and composition (Krawiec and Riley, 1990). Disruption of this inherent structure is usually detrimental to the organism. On the other hand it has been the traditional thinking that:

> "plasmid genes seem to be arranged so as to ensure hereditary stability and at the same time allow great structural flexibility ... plasmids can acquire new genes and grossly rearrange old ones to maintain a store of genetic information consistent with the need of their current host organism without compromising their own replication efficiency." (Novick, 1980)

This thinking may not be totally correct. Indeed, plasmids may contain an inherent structural organization and disruption of this organization could lead to plasmid instability. If the observed loss of tetracycline resistance and the inability of the plasmids to cotransfer markers is a reflection of instability then this may suggest that the acquisition of the additional DNA segment to form the R-prime disrupted some kind of basic genetic organization of R68.45. Loss of plasmid markers has been previously associated with the transfer of chromosomal markers (Haas and Holloway, 1978) and the formation of R-

primes (Morgan, 1982) by R68.45.

The loss of the DNA fragment associated with the 6.5 kb *Hind*III/*Sal*I fragment may also reflect the tendency of these plasmids to return to a basic structural organization. The 6.7 kb fragment from the native plasmid, R68.45, contains the $(IS21)_2$ sequence (Leemans et al., 1980). R68.45 was generated by the duplication of a 2.1 kb fragment on R68 to form $(IS21)_2$. Spontaneous loss of the 2.1 kb DNA fragment has been associated with R68.45 instability (Currier and Morgan, 1982; Haas and Riess, 1983). The loss of the 2.1 kb fragment would return R68.45 to a more basic and perhaps more stable form, namely R68.

Summary

The broad-host-range plasmid R68.45 possesses many attributes which make it an attractive tool to use in the examination of bacterial genetics. However, the use of this plasmid in prokaryotic molecular biology is limited. The limitation to its use is mainly due to the difficulty encountered when attempting to adapt basic molecular biology techniques to this plasmid.

Some major achievements of this work have been the ability to overcome some of the difficulties associated with working with the plasmid. One achievement was the ability

to isolate the plasmid consistently and in high yields. The ability to isolate large quantities of purified plasmid allowed the introduction of pulsed field gel electrophoresis to the restriction analysis of the plasmid.

Other noteworthy achievements of the project were the reproducible nature of the matings and the effective selective regimes in the interspecific and intergeneric matings followed by physical characterization to support the genetic interpretations.

One particular attribute of R68.45 which makes it attractive to use in bacterial genetics is the ability of the plasmid to form primes. Several primes were isolated using interspecific matings. The prime plasmids exhibited instability upon transfer during intergeneric crosses. The instability of the R-primes may limit their use as *in vivo* cloning vectors and have raised some interesting question concerning plasmid structure. However, the fundamental question concerning R-primes still remains: what is the mechanism of their formation. The techniques advanced in this study may allow for the further examination of this important question.

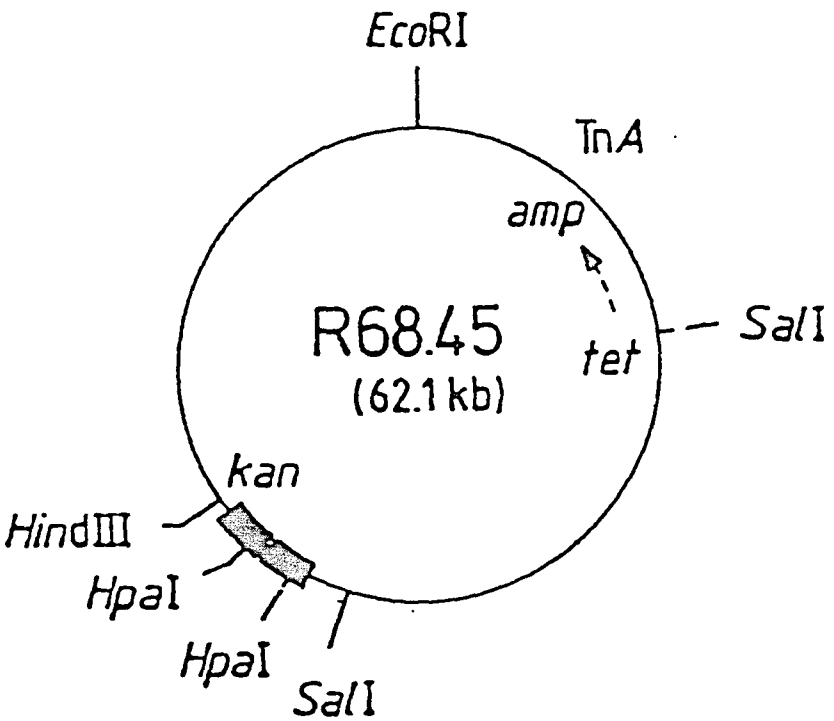

Figure 1. Physical map of R68.45. The map shows antibiotic resistance factors used as selective markers for R68.45: kanamycin (kan), tetracycline (tet), ampicillin/carbenicillin (amp). Also shown are the restriction enzyme cleavage sites used to characterize the plasmid. Figure taken from Reimmann and Haas (1987).

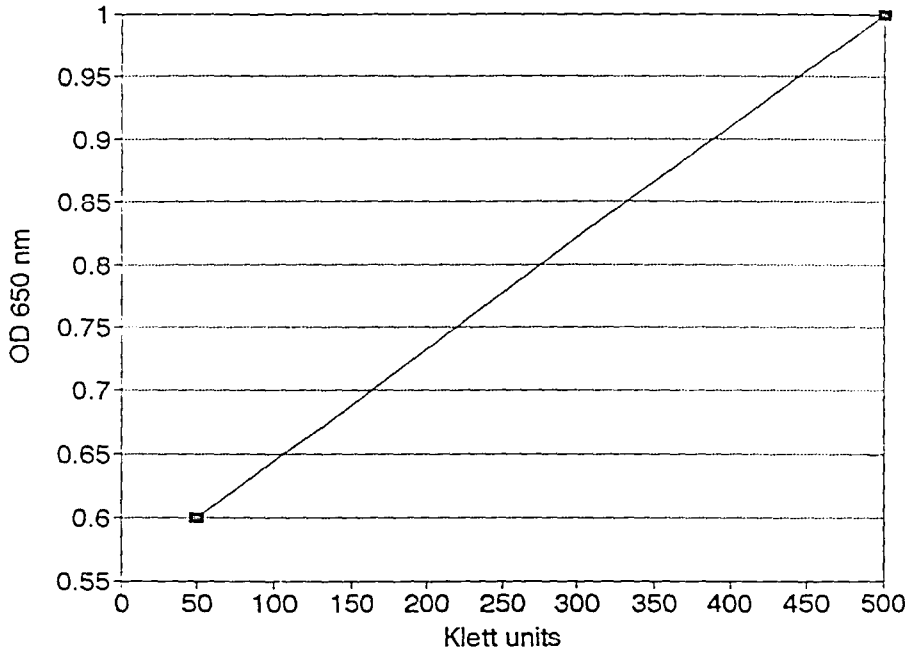

Figure 2: Graph for the conversion of the OD_{650}-nm to klett units. The graph was used to calculate the amount of TE used to resuspend the cell pellet during plasmid isolation. An OD_{650}-nm of 0.6 equals 1.5×10^8 cfu/ml, this is equivalent to 45 klett units. One absorbence unit is equal to 500 klett units.

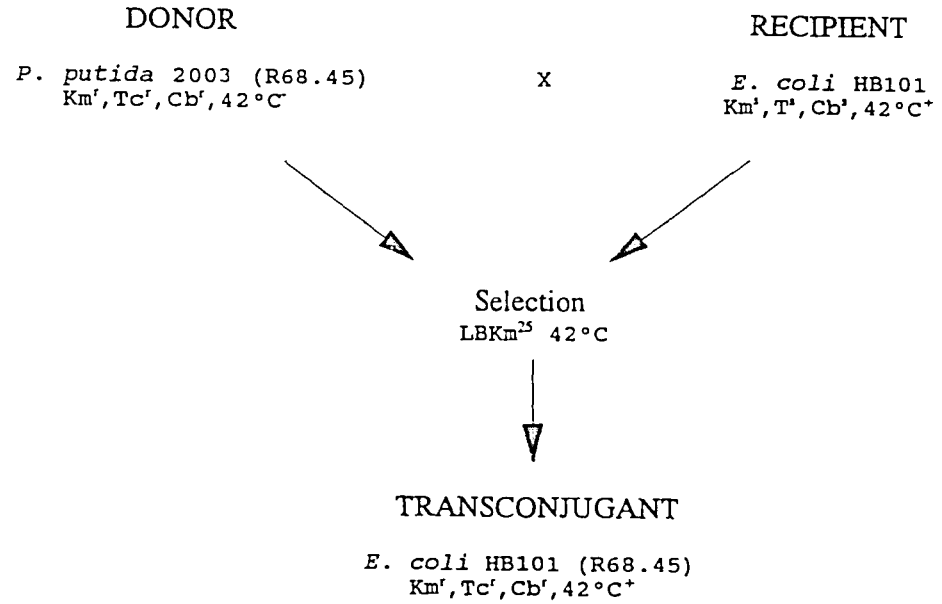

Figure 3. Outline of the bacterial mating between *P. putida* 2003 and *E. coli* HB101. Relevant phenotypes are listed below the organism. The selective conditions included growth on LB plates supplemented with kanamycin with incubation at 42° C.

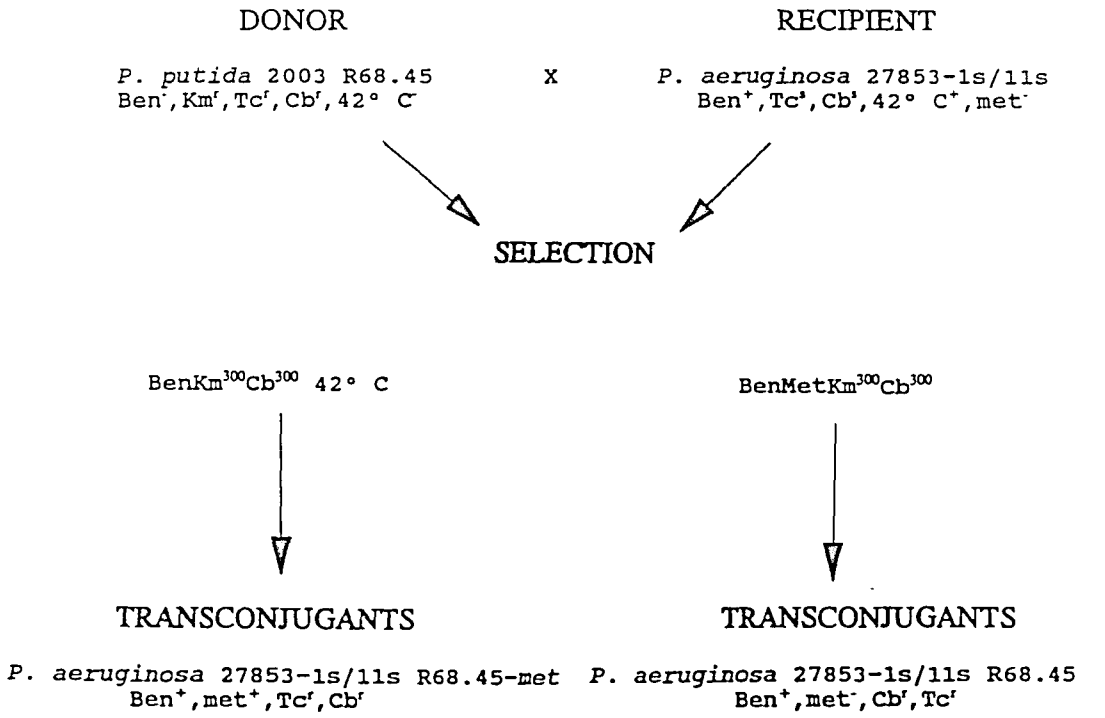

Figure 4. Outline of the bacterial mating between *P. putida* 2003 and *P. aeruginosa* 27853-1s or *P. aeruginosa* 27853-11s. The relevant phenotypes are listed below the organism. Selection for transconjugants was based on growth on benzoic acid plates supplemented with methionine, kanamycin, and carbenicillin incubated at 42° C. Selection for transconjugants containing R-primes was based on the same selective conditions exept that methionine was ommitted.

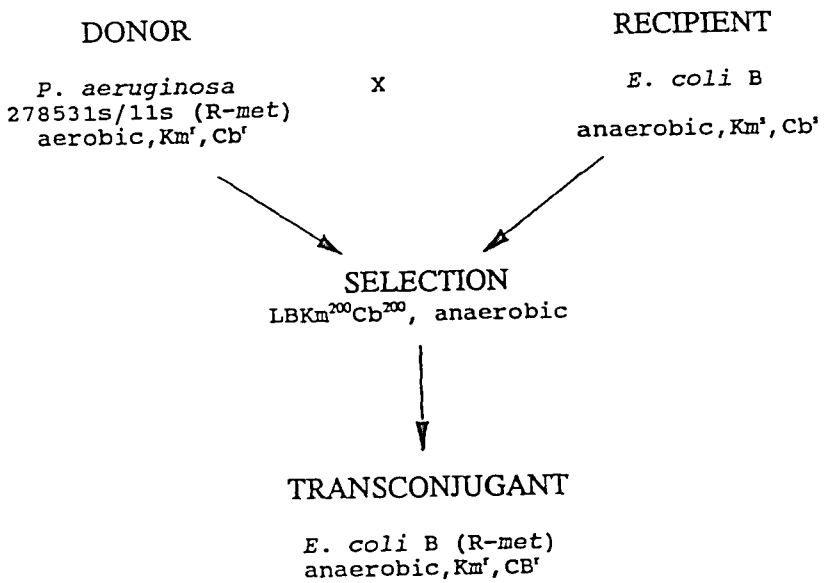

Figure 5. Outline of the bacterial mating between *P. aeruginosa* 27853-1s (R68.45-*met*) or 27853-11s (R68.45-*met*) and *E. coli* B. The relevant phenotypes are listed below the organism. Selection was based on growth on LB plates supplemented with kanamycin and carbenicillin placed under anaerobic conditions.

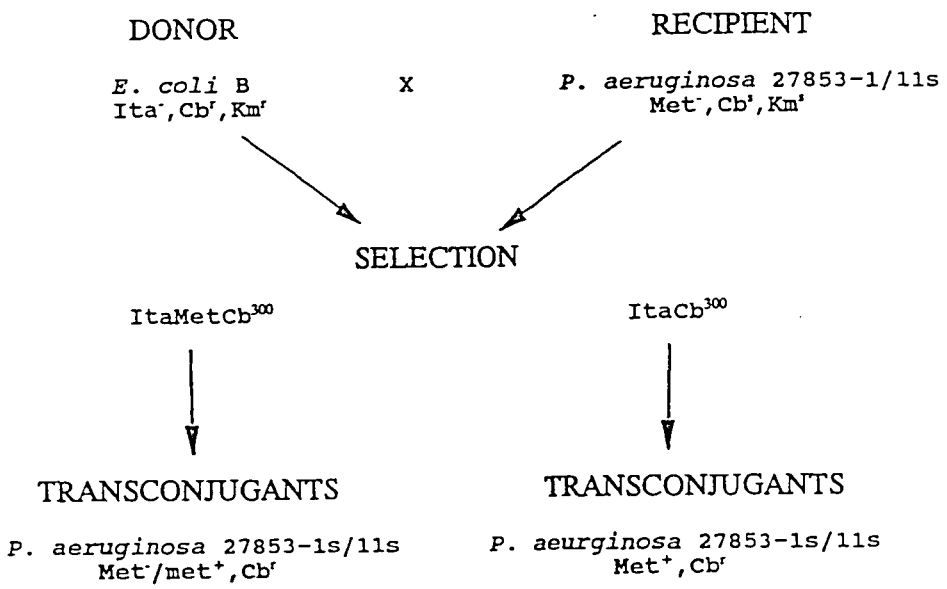

Figure 6. Outline of the mating between *E. coli* B containing the R68.45-*met* R-primes and *P. aeruginosa* 27853-1s or *P. aeruginosa* 27853-11s. Relevant phenotypes are listed below the organism. Selection was based on growth on itaconic acid plates supplemented with carbenicillin, or itaconic acid plates supplemented with carbenicillin and methionine.

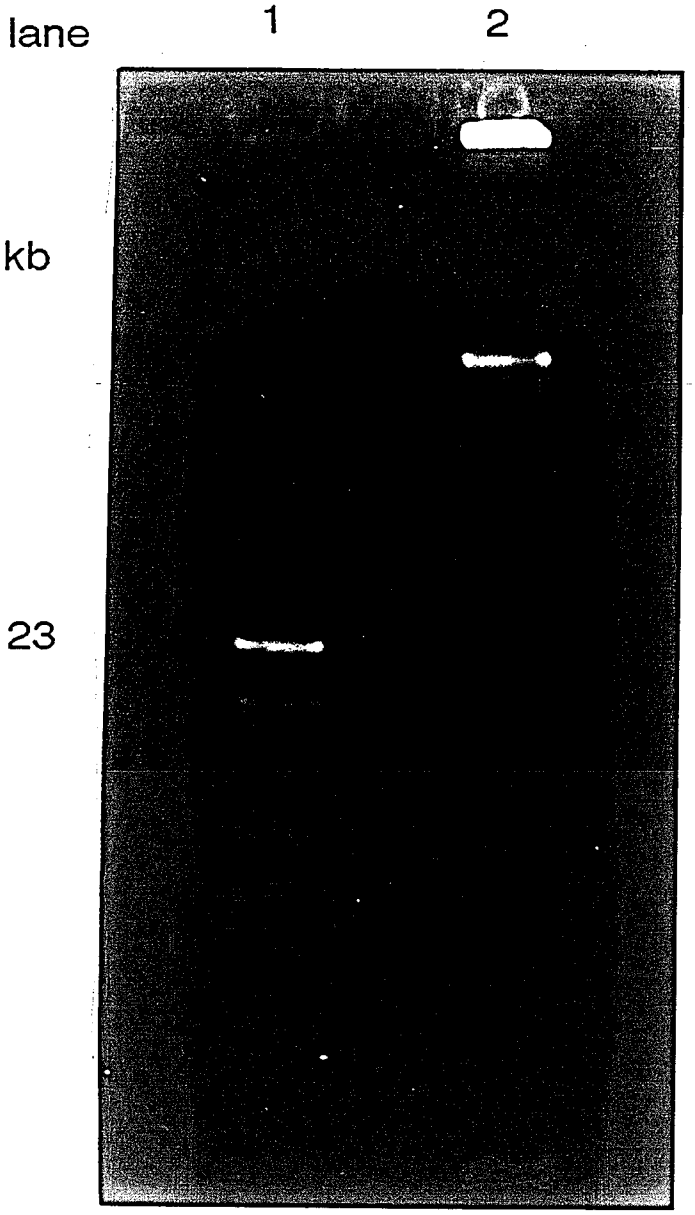

Figure 7. Plasmid isolation from *P. putida* 2003. Plasmid DNA isolated from *P. putida* 2003 was separated on a 1% agarose gel, then visualized using ethidium bromide. (lane 1 λ Hind*III* marker, lane 2 - *P. putida* 2003).

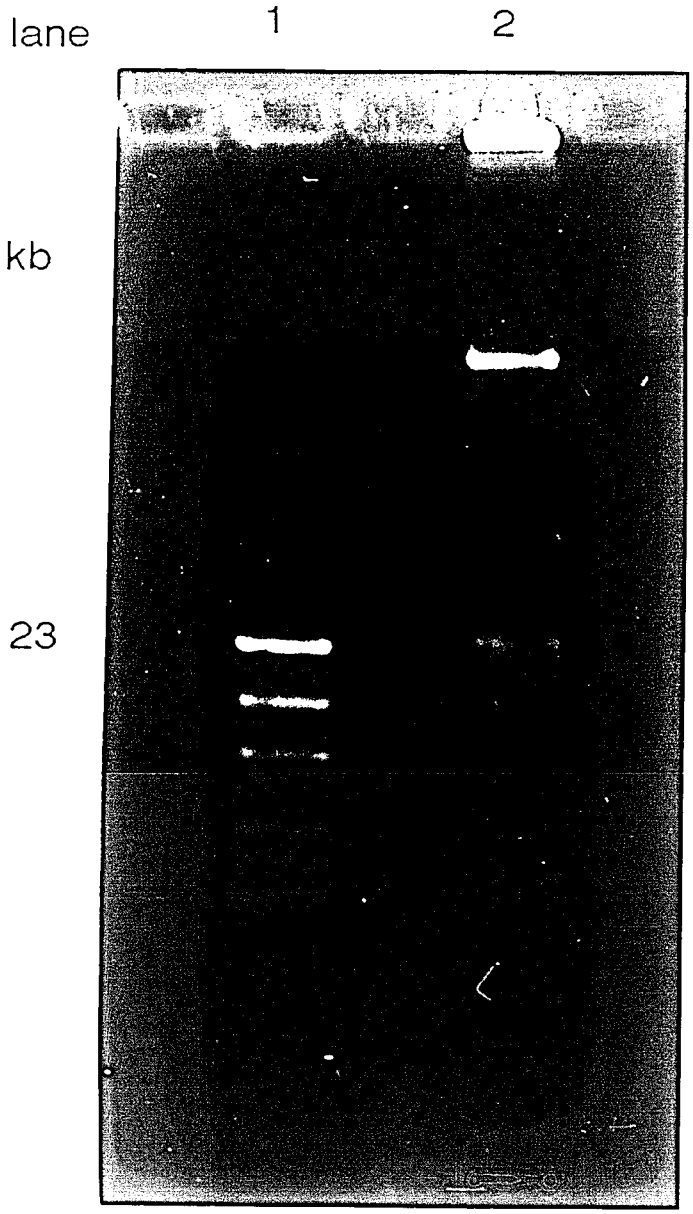

Figure 7. Plasmid isolation from *P. putida* 2003. Plasmid DNA isolated from *P. putida* 2003 was separated on a 1% agarose gel, then visualized using ethidium bromide. (lane 1 λ Hind*III* marker, lane 2 - *P. putida* 2003).

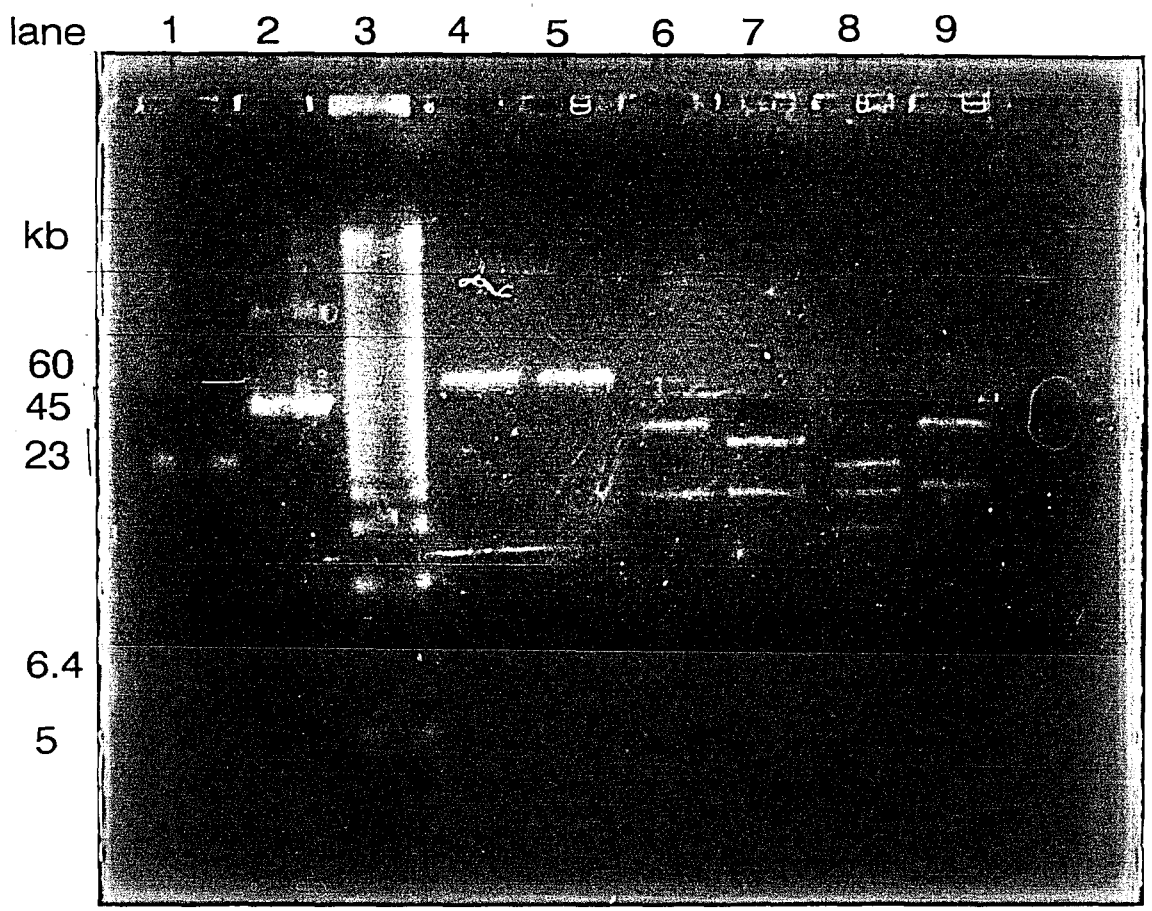

Figure 8. Restriction digests of the plasmid isolated from *P. putida* 2003. Plasmid DNA was restricted with *Eco*RI, *Hind*III, *Sal*I, and combinations of these. The restriction fragments were resolved using pulsed field gel electrophoresis (130 V/cm, 18 hr, 1-10 sec ramp). (lane 1 - λ *Hind*III, lane 2 - λ uncut, lane 3 - 5 kb ladder, lane 4 - *Hind*III, lane 5 - *Eco*RI, lane 6 - *Sal*I, lane 7 - *Sal*I and *Hind*III, lane 8 - *Eco*RI and *Sal*I, lane 9 - *Eco*RI and *Hind*III).

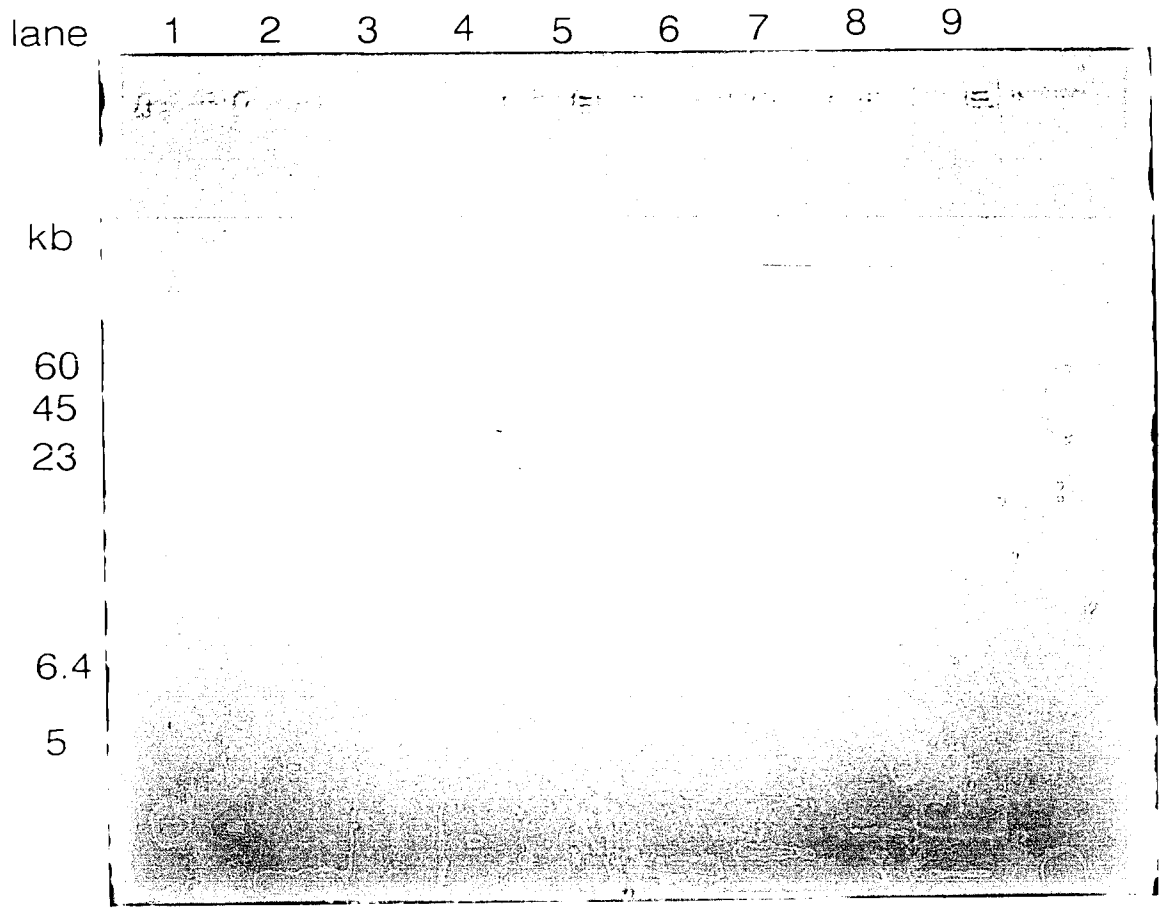

Figure 8. Restriction digests of the plasmid isolated from *P. putida* 2003. Plasmid DNA was restricted with *Eco*RI, *Hin*dIII, *Sal*I, and combinations of these. The restriction fragments were resolved using pulsed field gel electrophoresis (130 V/cm, 18 hr, 1-10 sec ramp). (lane 1 - λ *Hin*dIII, lane 2 - λ uncut, lane 3 - 5 kb ladder, lane 4 - *Hin*dIII, lane 5 - *Eco*RI, lane 6 - *Sal*I, lane 7 - *Sal*I and *Hin*dIII, lane 8 - *Eco*RI and *Sal*I, lane 9 - *Eco*RI and *Hin*dIII).

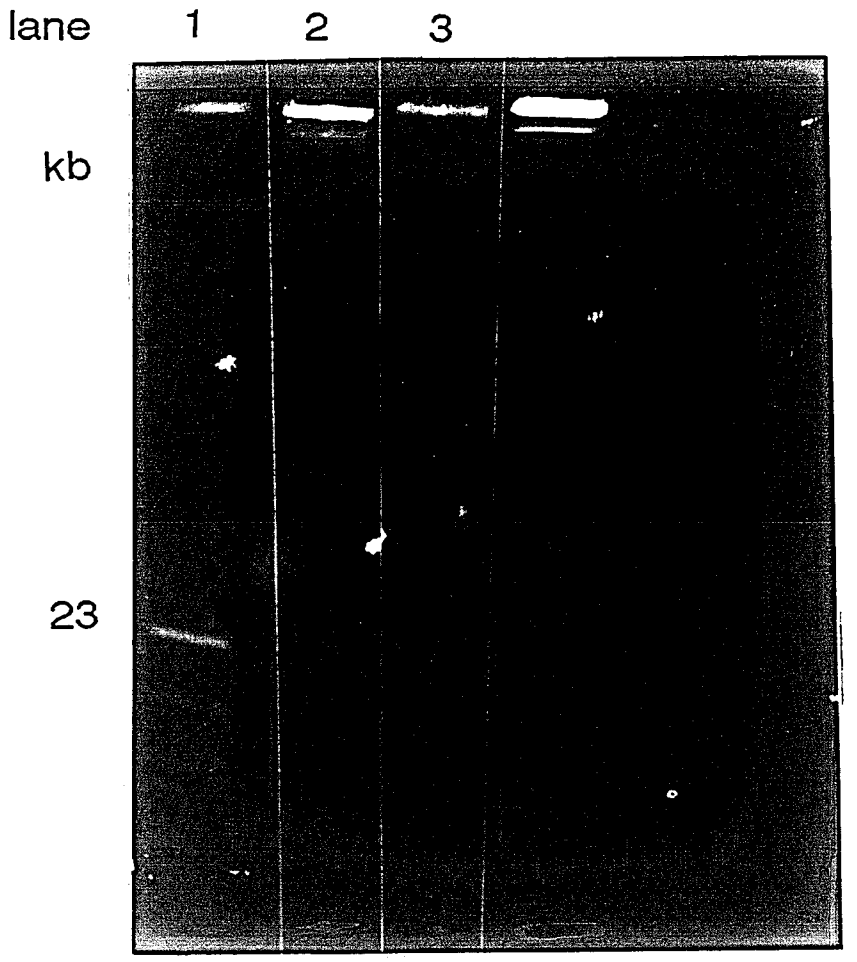

Figure 9. Plasmid preparations of *P. putida* 2003 and *P. putida* 2003b$_2$. Plasmid preparations from *P. putida* 2003 and *P. putida* 2003b$_2$ were electrophoresed through a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. putida* 2003b$_2$).

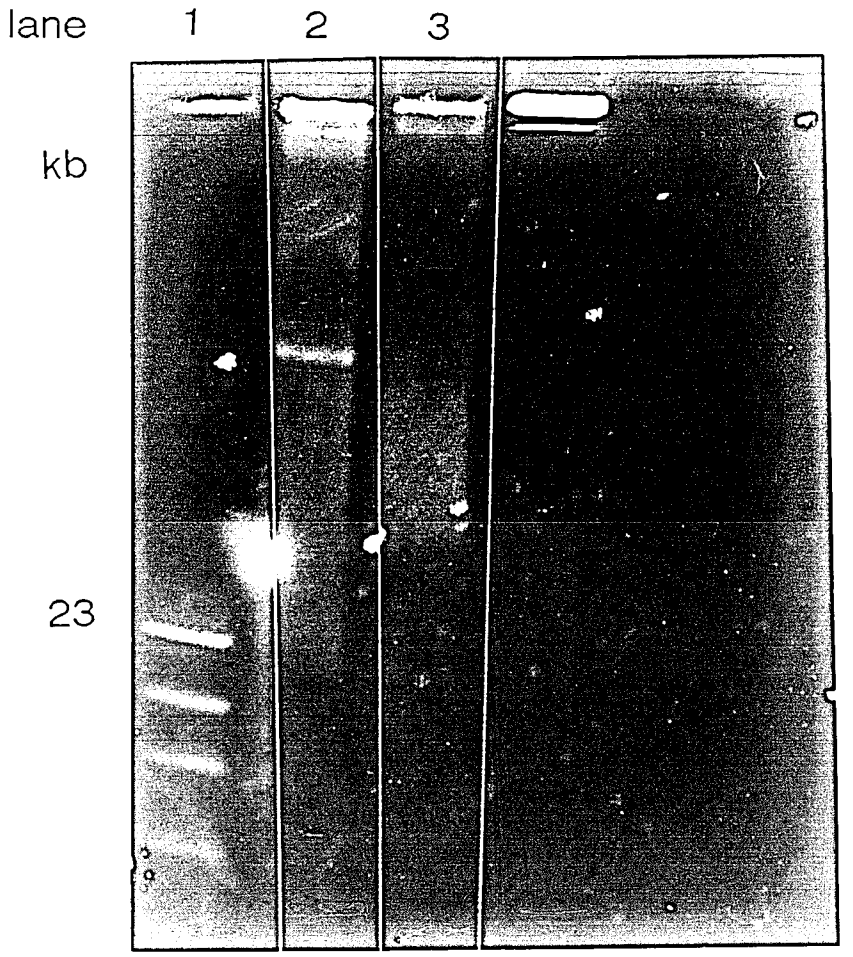

Figure 9. Plasmid preparations of *P. putida* 2003 and *P. putida* 2003b. Plasmid preparations from *P. putida* 2003 and *P. putida* 2003b were electrophoresed through a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. putida* 2003b).

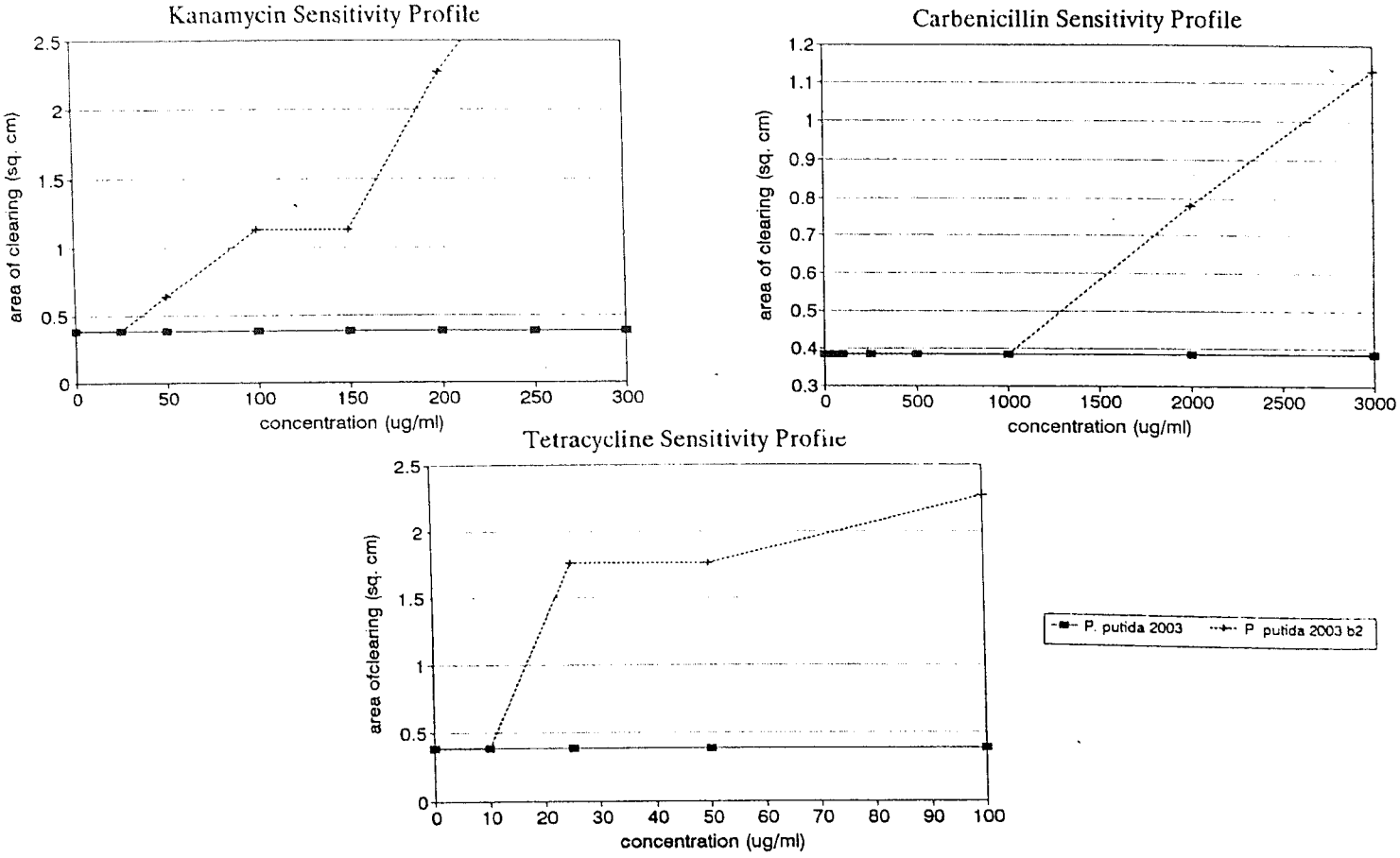

Figure 10. Antibiotic profile of *P. putida* 2003 and *P. putida* 2003b$_2$. The graphs represent the area of clearing vs. the concentration of antibiotic. The area of clearing is a measure of antibiotic sensitivity exhibited by the test organism.

37853 IS Fig 24

MICROLOG (TM) 1, RELEASE 2.00

date : 10/18/91
plate type : GN
plate # : 12
hour : 24

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, first ID choice positive > 90% of time
XX- = positive, first ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	..	..	XX	XX	..	XX	..	..	..	..
B	..	XX	..	..	..	XX	..	..	..	B-	XX	B-
C	..	..	XX	..	..	..	..	..	..	..	XX	XX
D	XX	XX	XX	XX	..	..	XX	..	..	XX	XX	..
E	..+	XX	XX	XX	..	XX	XX	XX	XX	XX-	XX	XX
F	XX	XX	..	..	XX	XX	XX	XX	XX	XX	..	..
G	XX	XX	..+	XX	..	XX	XX	B	XX	XX	XX	XX
H	XX	XX	..	..	..	XX	XX	..	XX	..	..	..

BIO-NUMBER : 0320-2107-1003-7446-3577-6374-6577-6150

GOOD IDENTIFICATION : PSEUDOMONAS AERUGINOSA

BEST SPECIES :	SIM	DIST	AVG	MAX
1) PSEUDOMONAS AERUGINOSA	0.751	3.418	2.125	4.412
2) PSEUDOMONAS FLUORESCENS SUBGROUP C	0.001	5.658	3.000	6.688
3) PSEUDOMONAS PUTIDA SUBGROUP A	0.000	8.074	2.781	6.744
4) PSEUDOMONAS-UNIDENTIFIED FLUORESCENT	0.000	9.738	1.938	5.387
5) PSEUDOMONAS FRAGI	0.000	10.588	3.271	8.019
6) PSEUDOMONAS CITRONELLOLIS	0.000	11.048	1.250	6.300
7) PSEUDOMONAS MENDOCINA	0.000	11.120	2.375	6.188
8) PSEUDOMONAS PUTIDA SUBGROUP B	0.000	11.265	3.042	5.856
9) PSEUDOMONAS PSEUDOALCALIGENES SUBGROUP A	0.000	11.333	2.281	7.769
10) PSEUDOMONAS FLUORESCENS SUBGROUP B	0.000	12.086	1.406	4.544
other : ?	-----	-----	-----	-----

Figure 11. Representative Biolog printout. Biolog plates were used to confirm the species identification of the organism. Relevent Biolog data is shown in table 2.

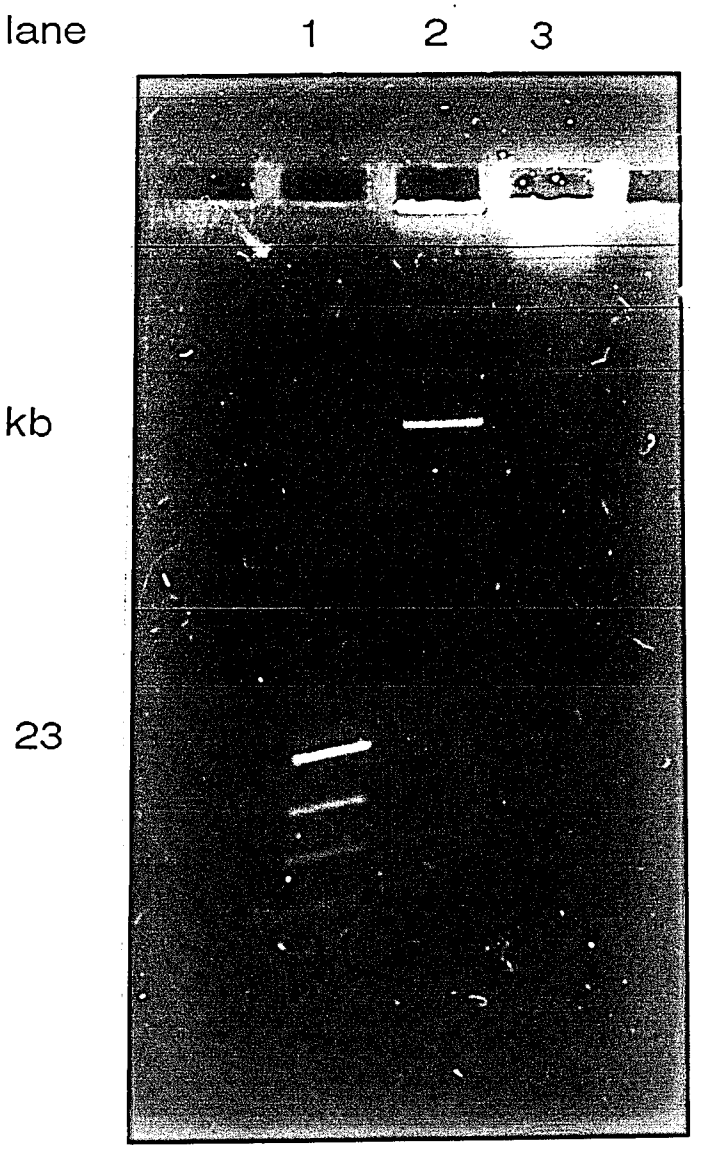

Figure 12. Plasmid analysis of *P. putida* 2003 and *E. coli* HB101. Plasmid preparations from *P. putida* 2003 and *E. coli* HB101 were separated on a 1% agarose gel. (lane 1 - λ Hind*III*, lane 2 - *P. putida* 2003, lane 3, *E. coli* HB101).

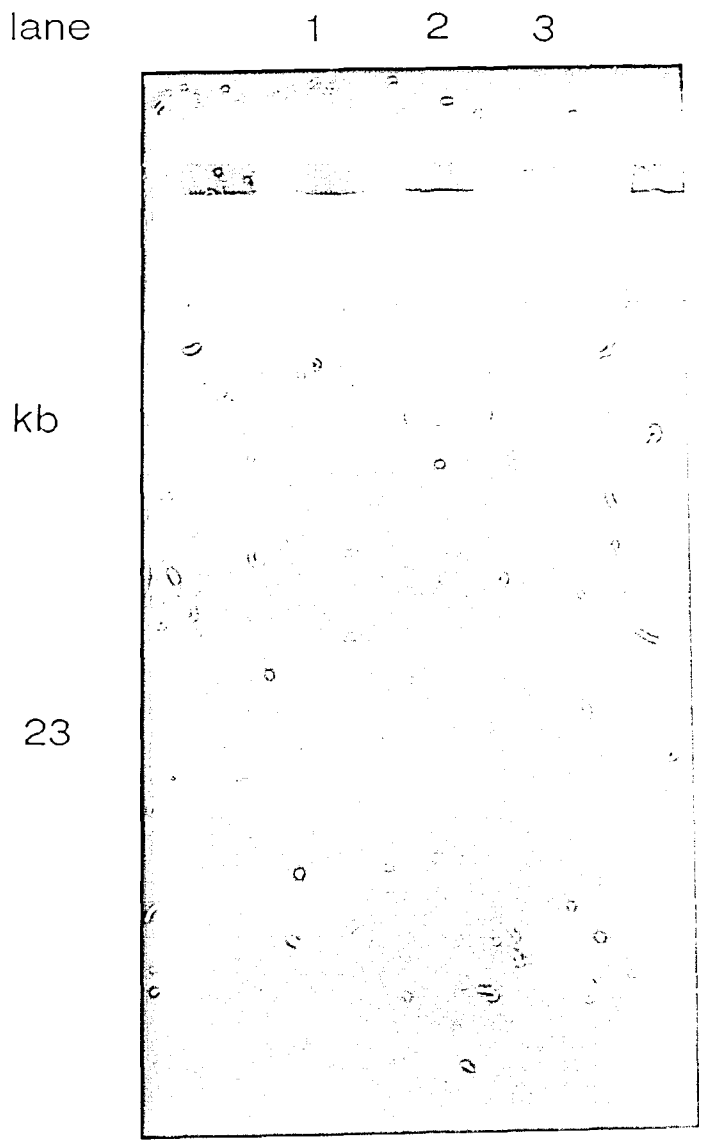

Figure 12. Plasmid analysis of *P. putida* 2003 and *E. coli* HB101. Plasmid preparations from *P. putida* 2003 and *E. coli* HB101 were separated on a 1% agarose gel. (lane 1 - λ *HindIII*, lane 2 - *P. putida* 2003, lane 3, *E. coli* HB101).

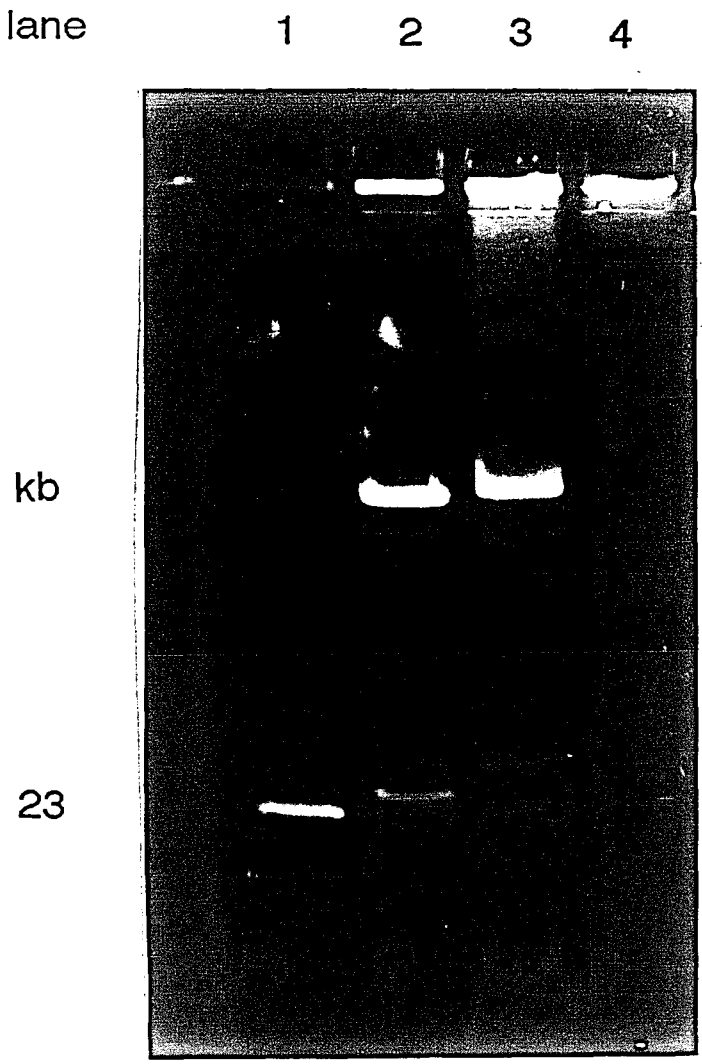

Figure 13. Plasmid isolations of *P. putida* 2003 and an *E. coli* HB101 transconjugant. Plasmid preparations of *P. putida* 2003 and an *E. coli* HB101 transconjugant were separated on a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *E. coli* HB101 transconjugant, lane 4 - *E. coli* HB101).

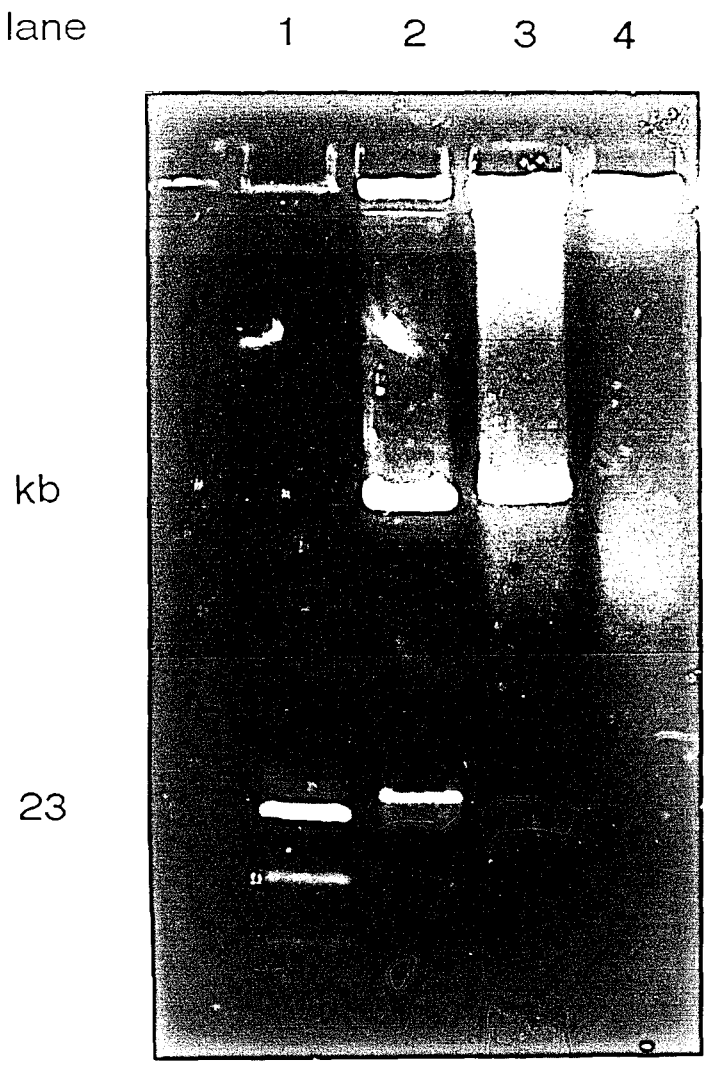

Figure 13. Plasmid isolations of *P. putida* 2003 and an *E. coli* HB101 transconjugant. Plasmid preparations of *P. putida* 2003 and an *E. coli* HB101 transconjugant were separated on a 1% agarose gel. (lane 1 - λ *Hin*dIII, lane 2 - *P. putida* 2003, lane 3 - *E. coli* HB101 transconjugant, lane 4 - *E. coli* HB101).

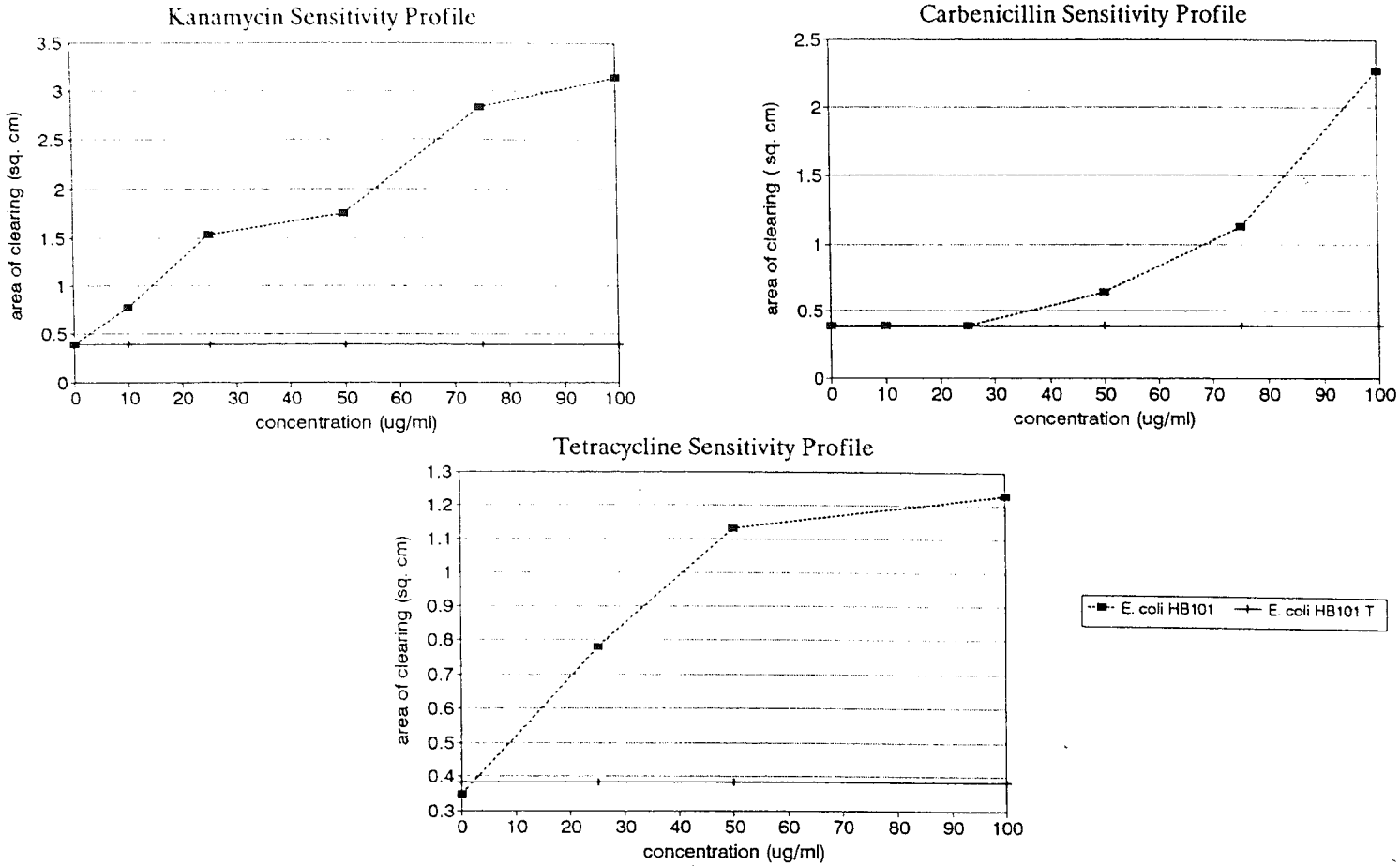

Figure 14. Antibiotic profile of *E. coli* HB101 and an *E. coli* HB101 transconjugant. The graphs represent the area of clearing vs. the concentration. The area of clearing is a measure of the antibiotic sensitivity of the test organisms.

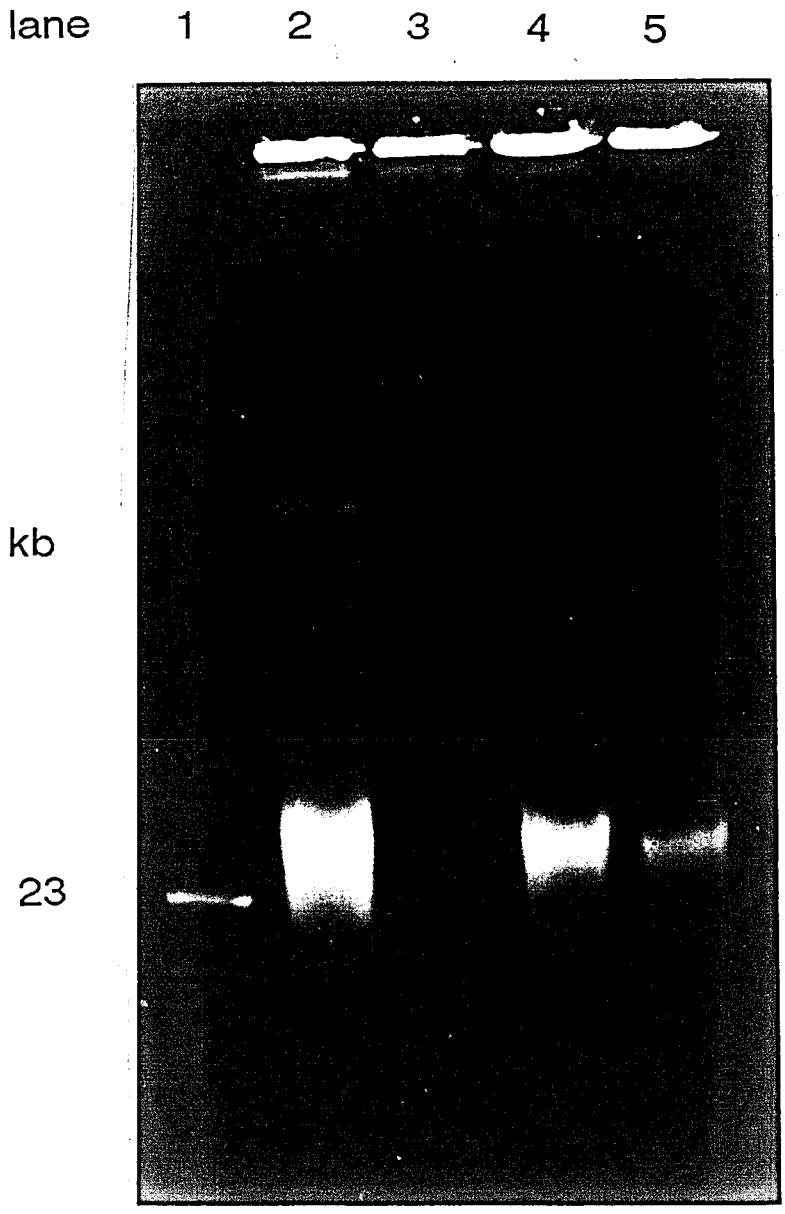

Figure 15. Plasmid preparations of *P. putida* 2003, *P. aeruginosa* 27853-1s, *P. aeruginosa* 27853-11s, and *P. aeruginosa* 27853. Plasmid DNA was electrophoresed through a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s, lane 4 - *P. aeruginosa* 27853-11s, lane 5 - *P. aeruginosa* 27853).

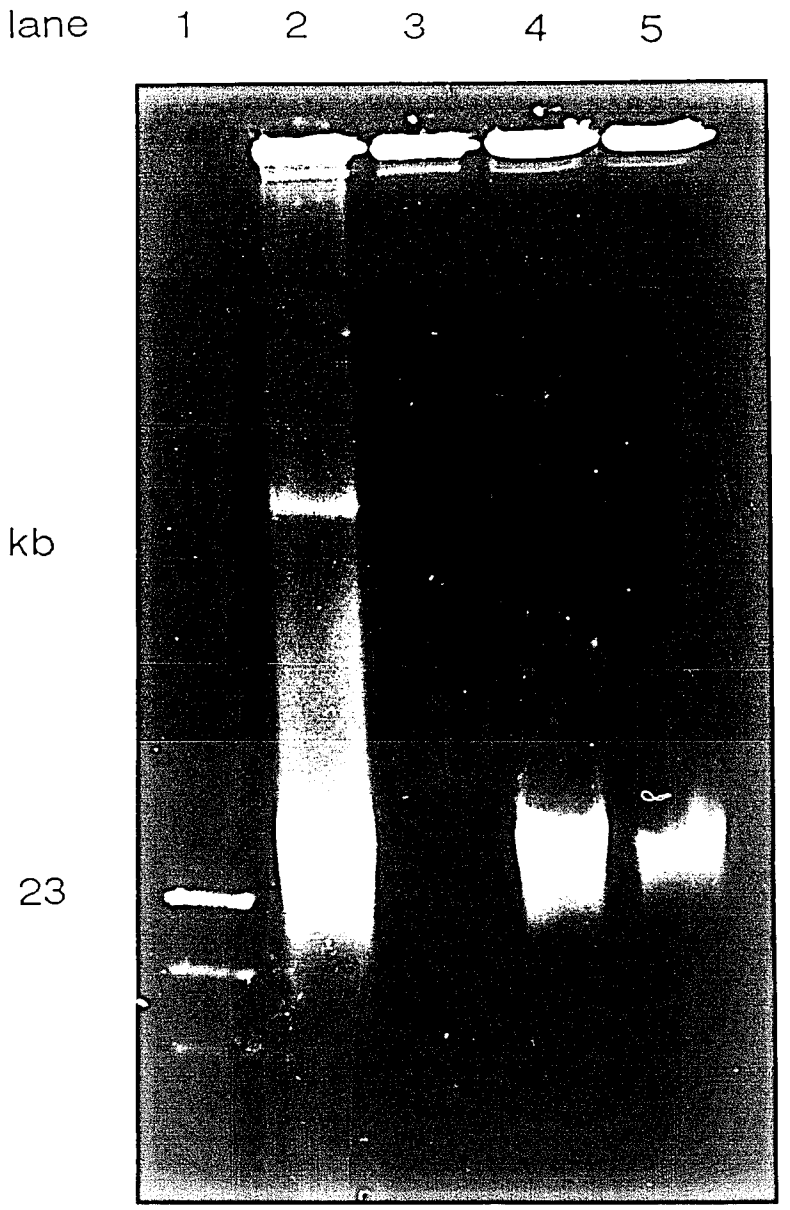

Figure 15. Plasmid preparations of *P. putida* 2003, *P. aeruginosa* 27853-1s, *P. aeruginosa* 27853-11s, and *P. aeruginosa* 27853. Plasmid DNA was electrophoresed through a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s, lane 4 - *P. aeruginosa* 27853-11s, lane 5 - *P. aeruginosa* 27853).

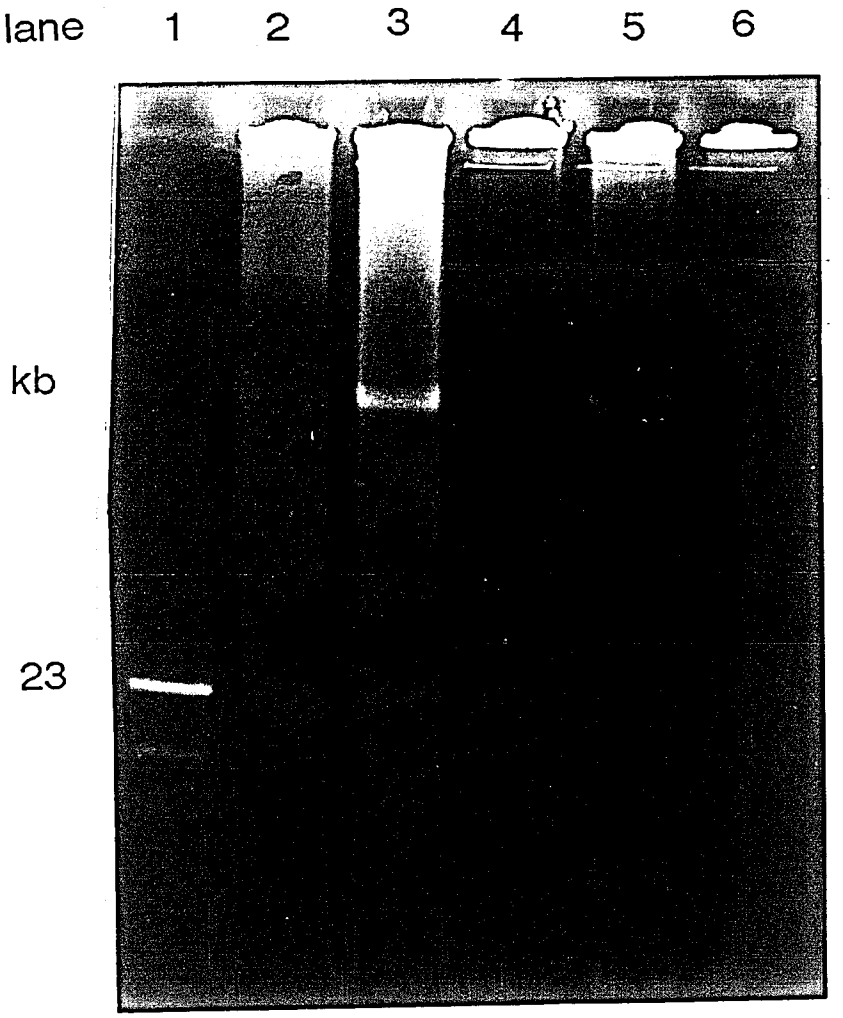

Figure 16. Plasmid isolation of *P. aeruginosa* 27853-1s and 27853-11s transconjugants. Plasmids isolated from *P. aeruginosa* transconjugants were separated on a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s (R68.45), lane 4 - *P. aeruginosa* 27853-1s, lane 5 - *P. aeruginosa* 27853-11s (R68.45), lane 6 *P. aeruginosa* 27853-11s).

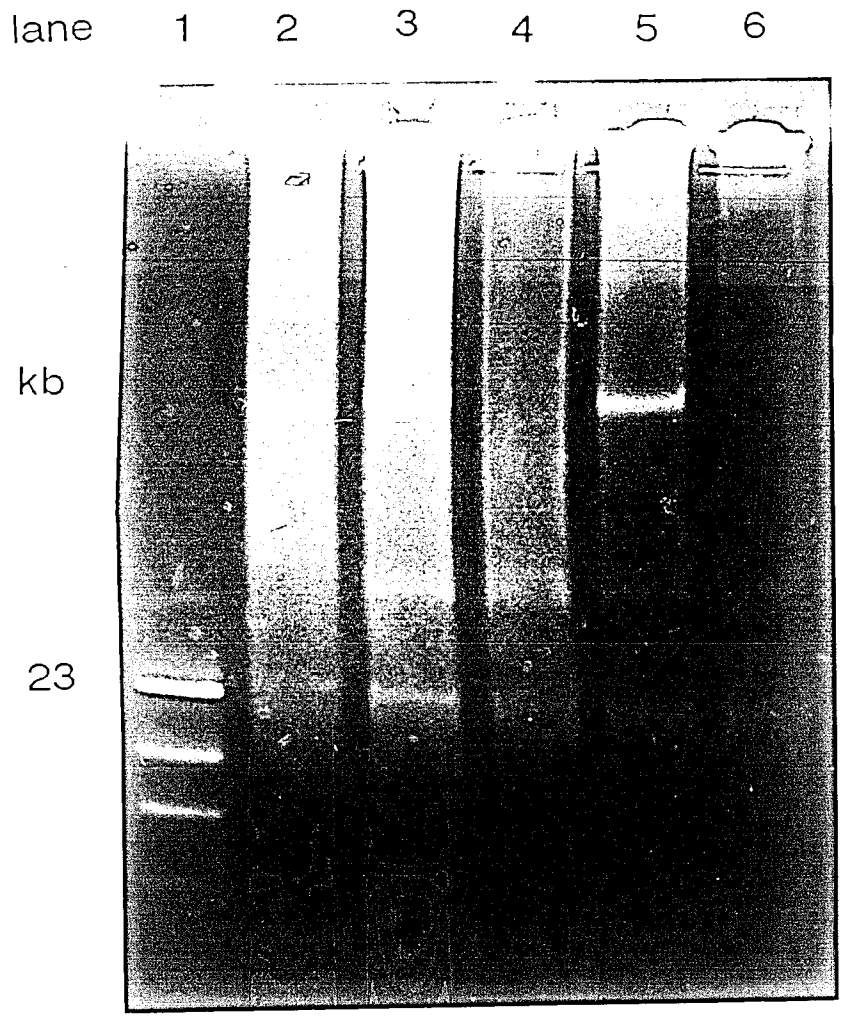

Figure 16. Plasmid isolation of *P. aeruginosa* 27853-1s and 27853-11s transconjugants. Plasmids isolated from *P. aeruginosa* transconjugants were separated on a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s (R68.45), lane 4 - *P. aeruginosa* 27853-1s, lane 5 - *P. aeruginosa* 27853-11s (R68.45), lane 6 *P. aeruginosa* 27853-11s).

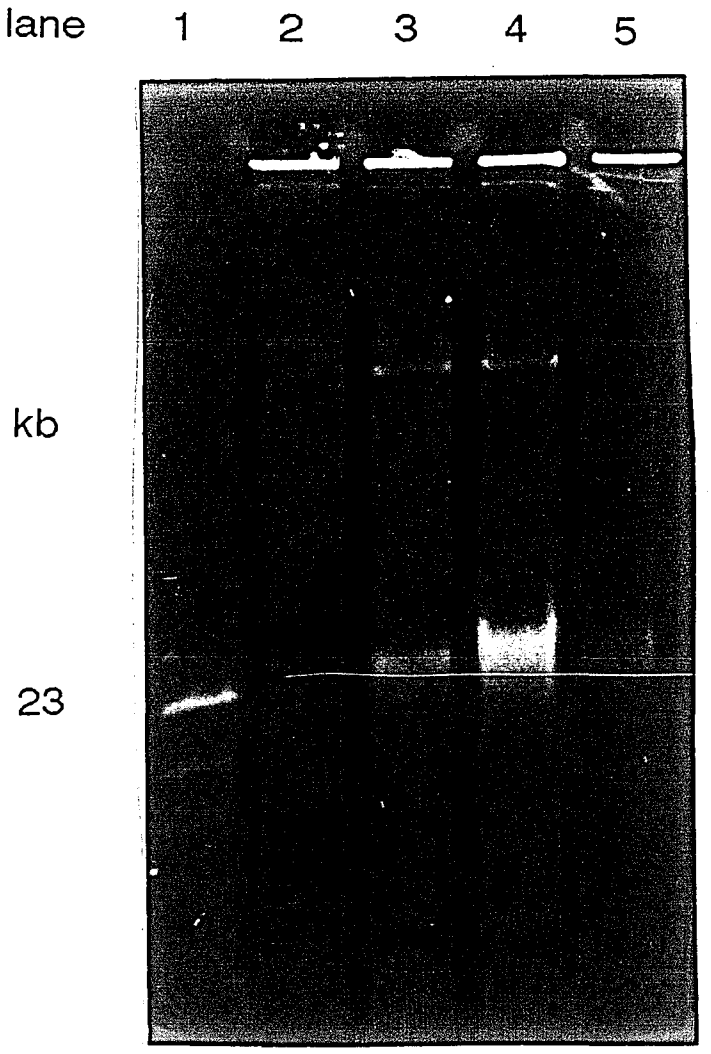

Figure 17. Prime plasmids isolated from *P. aeruginosa* 27853-1s transconjugants. Plasmid DNA isolated from *P. aeruginosa* 27853-1s transconjugants containing R-*met* primes (first mating). (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s pPM13, lane 4 - *P. aeruginosa* 27853-1s pPM15, lane 5 - *P. aeruginosa* 27853-1s).

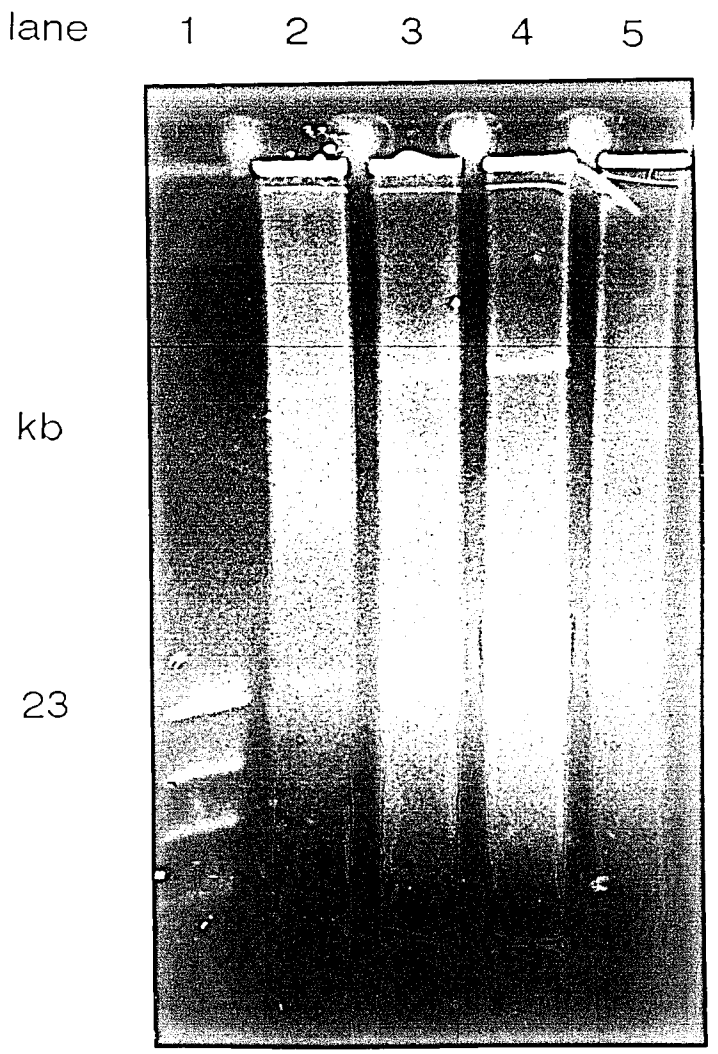

Figure 17. Prime plasmids isolated from *P. aeruginosa* 27853-1s transconjugants. Plasmid DNA isolated from *P. aeruginosa* 27853-1s transconjugants containing R-met primes (first mating). (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s pPM13, lane 4 - *P. aeruginosa* 27853-1s pPM15, lane 5 - *P. aeruginosa* 27853-1s).

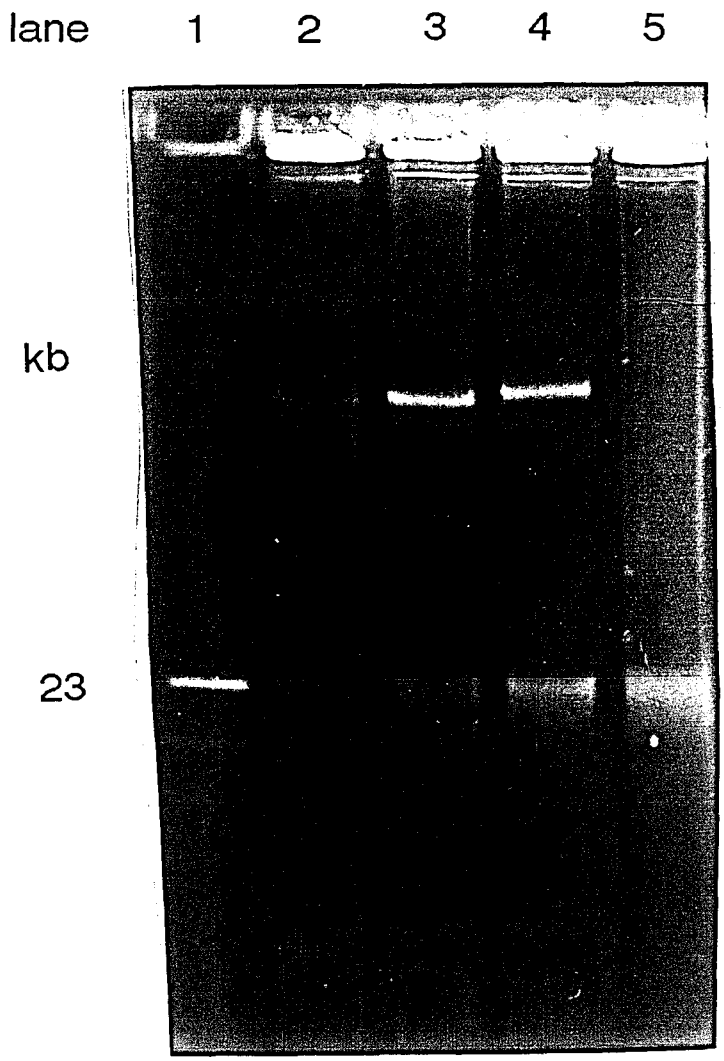

Figure 18. Prime plasmids isolated from *P. aeruginosa* 27853-1s transconjugants second mating.
Plasmid DNA isolated from *P. aeruginosa* 27853-1s transconjugants containing R-*met* primes. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s pPM5, lane 4 - *P. aeruginosa* 27853-1s pPM21, lane 5 - *P. aeruginosa* 27853-1s).

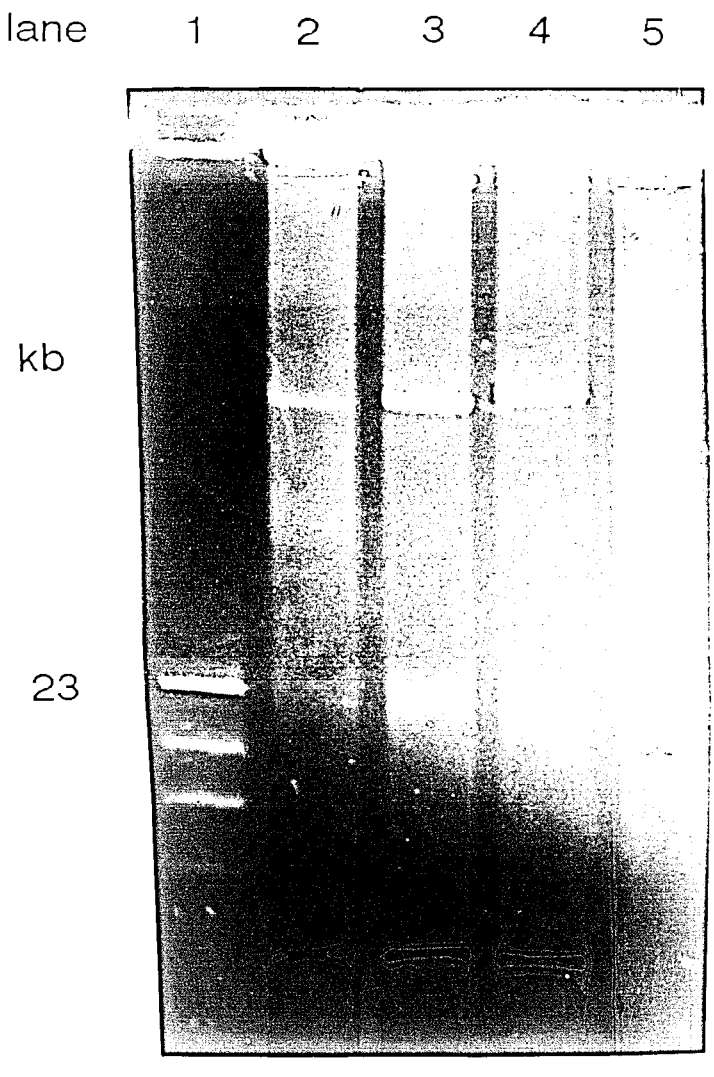

Figure 18. Prime plasmids isolated from *P. aeruginosa* 27853-1s transconjugants second mating.
Plasmid DNA isolated from *P. aeruginosa* 27853-1s transconjugants containing R-*met* primes. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-1s pPM5, lane 4 - *P. aeruginosa* 27853-1s pPM21, lane 5 - *P. aeruginosa* 27853-1s).

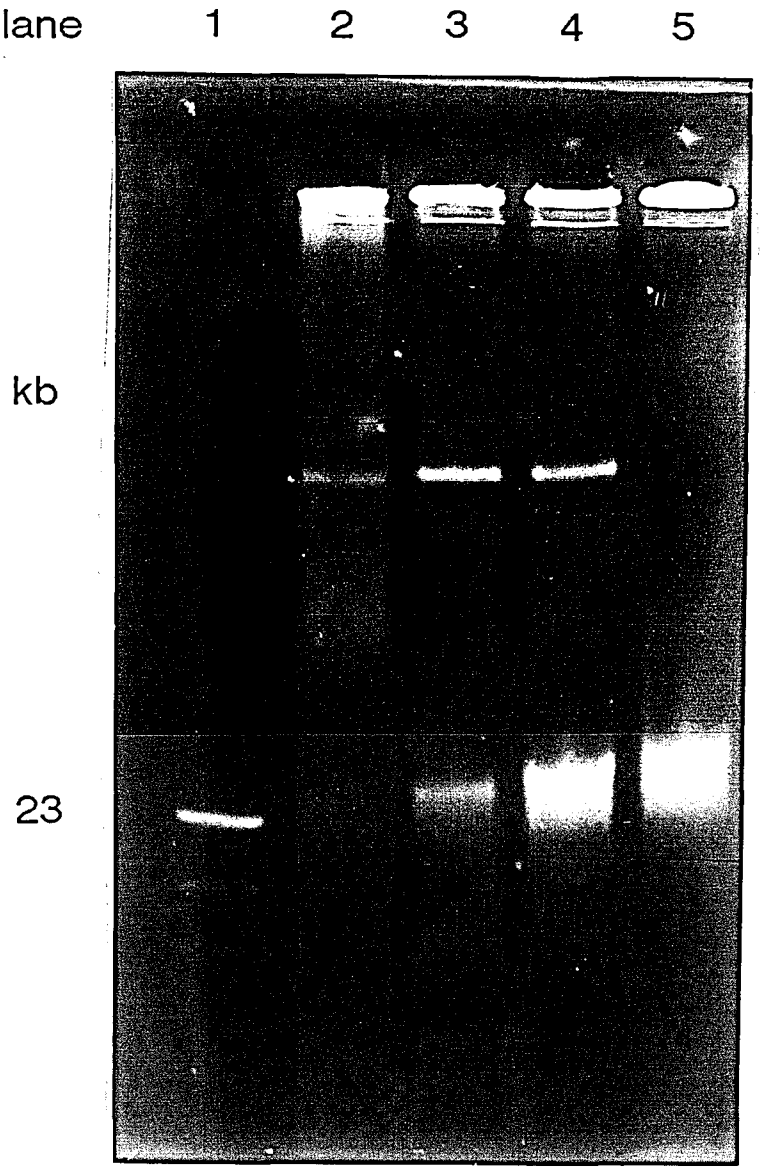

Figure 19. Prime plasmids isolated from *P. aeruginosa* 27853-11s transconjugants. Electrophoretic separation of plasmid DNA isolated from R-*met* containing *P. aeruginosa* transconjugants. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-11s pPM4, lane 4 - *P. aeruginosa* 27853-11s pPM7, lane 5 *P. aeruginosa* 27853-11s).

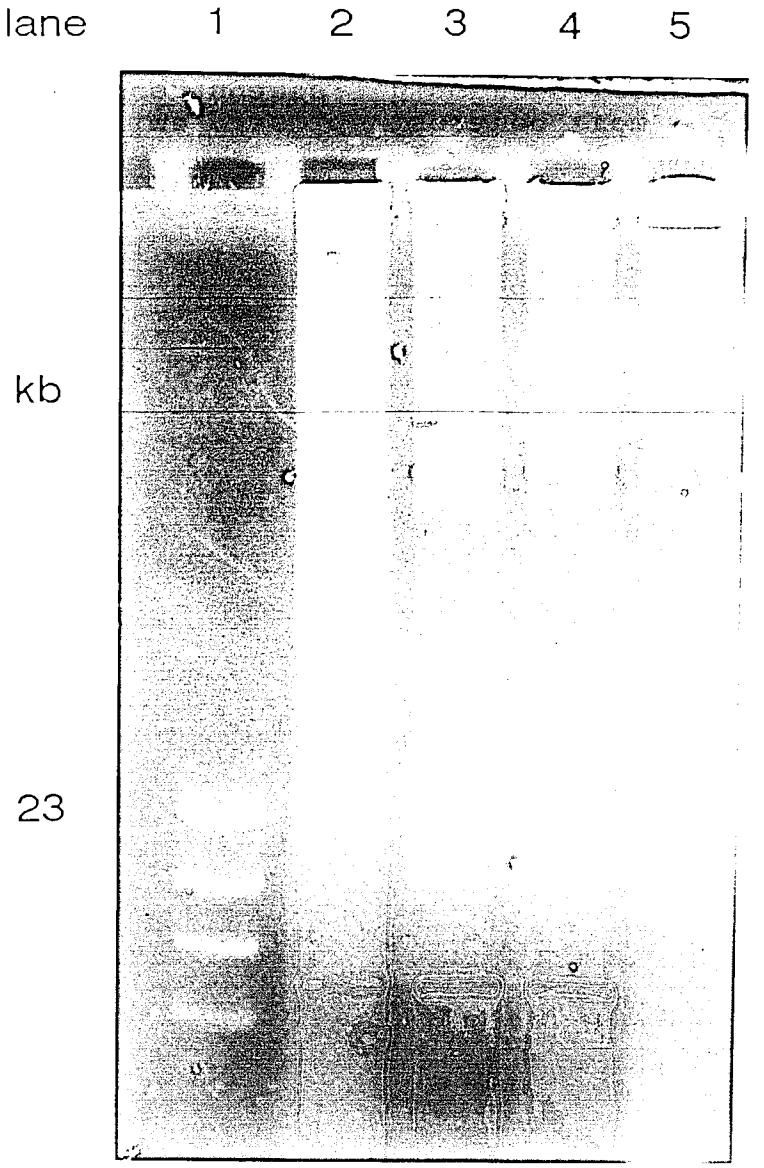

Figure 19. Prime plasmids isolated from *P. aeruginosa* 27853-11s transconjugants. Electrophoretic separation of plasmid DNA isolated from R-*met* containing *P. aeruginosa* transconjugants. (lane 1 - λ *Hind*III, lane 2 - *P. putida* 2003, lane 3 - *P. aeruginosa* 27853-11s pPM4, lane 4 - *P. aeruginosa* 27853-11s pPM7, lane 5 *P. aeruginosa* 27853-11s).

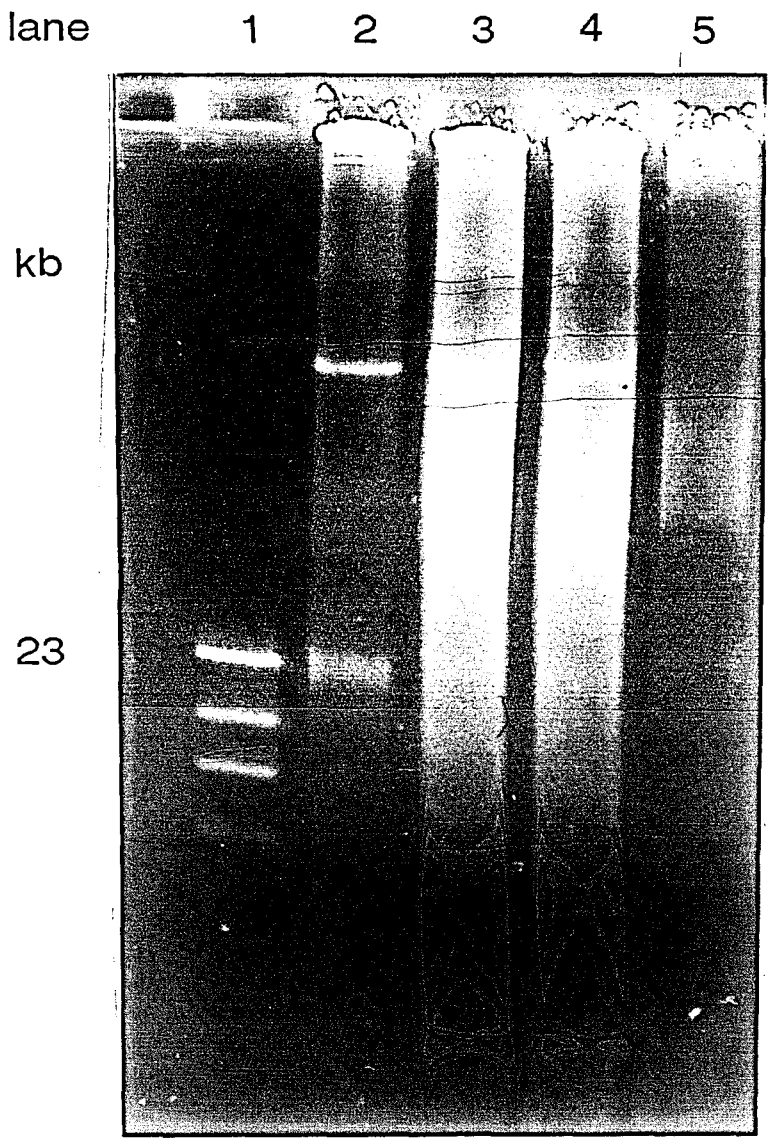

Figure 20: ***E. coli*** **B transconjugant containing the plasmid pPM4.** Plasmid DNA isolated from *E. coli* B transconjugants following the mating with *P. aeruginosa* 27853-11s pPM4. (lane 1- λ *Hind*III, lane 2 - *P. aeruginosa* 27853-11s pPM4, lane 3 and lane 4 - *E. coli* B transconjugants, lane 5 - *E. coli* B).

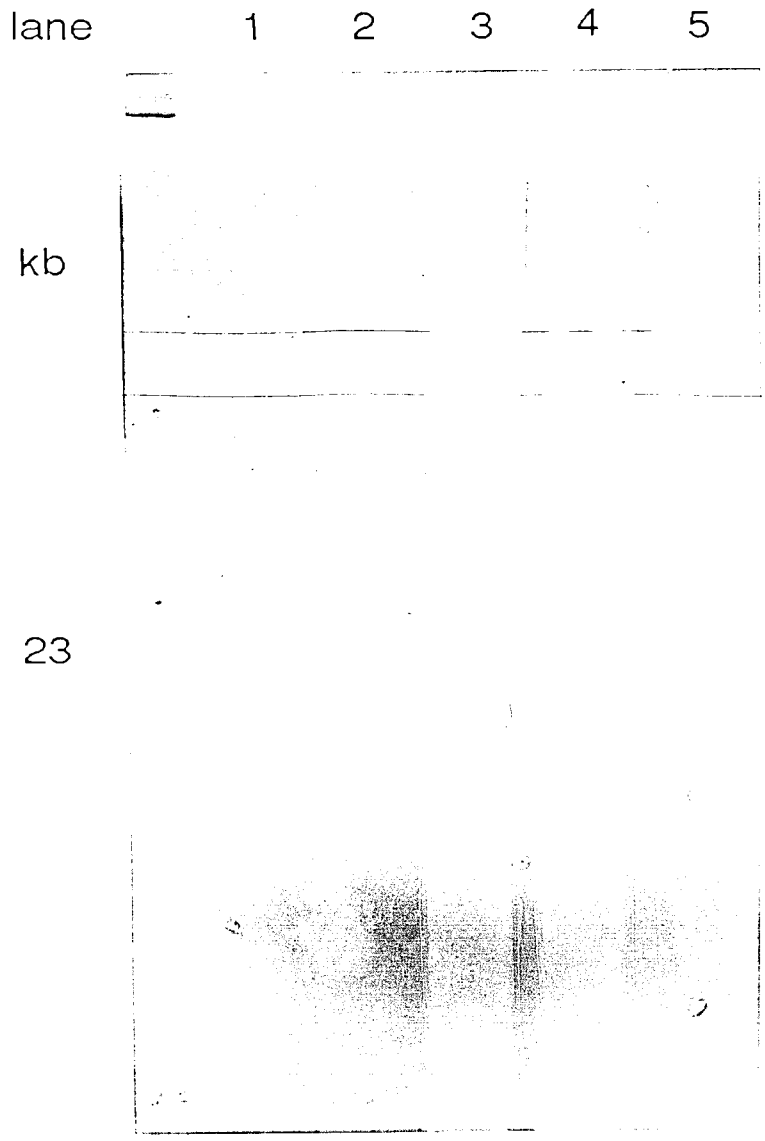

Figure 20: ***E. coli*** **B transconjugant containing the plasmid pPM4.** Plasmid DNA isolated from *E. coli* B transconjugants following the mating with *P. aeruginosa* 27853-11s pPM4. (lane 1- λ *Hind*III, lane 2 - *P. aeruginosa* 27853-11s pPM4, lane 3 and lane 4 - *E. coli* B transconjugants, lane 5 - *E. coli* B).

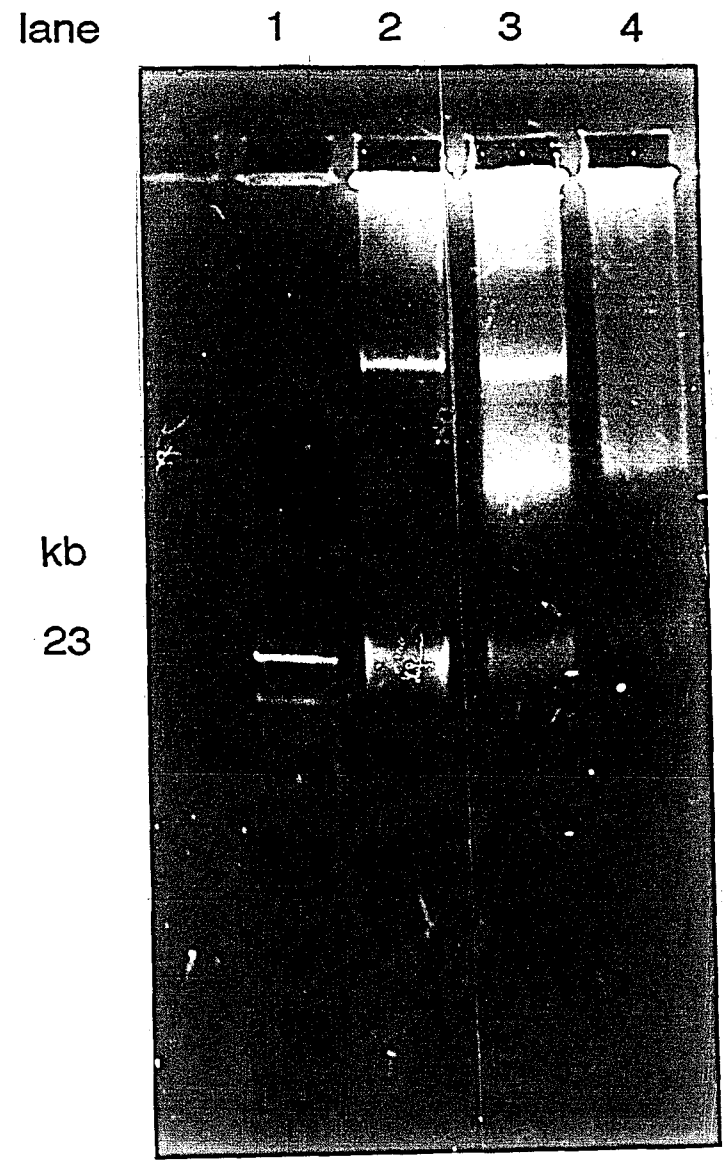

Figure 21. ***E. coli*** **B transconjugant containing the plasmid pPM21.** Plasmid DNA isolated from an *E. coli* B transconjugant following mating with *P. aeruginosa* 27853-1s pPM21. (lane 1 - λ *Hind*III, lane 2 - *P. aeruginosa* 27853-1s pPM21, lane 3 - *E. coli* B transconjugant, lane 4 - *E. coli* B).

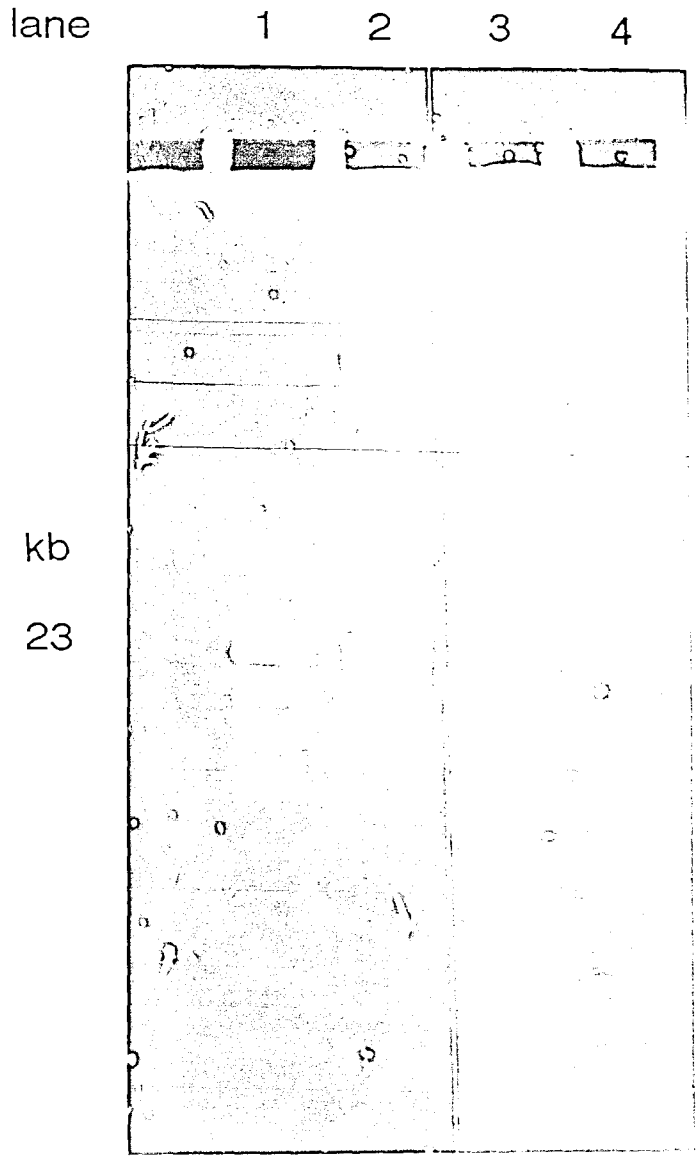

Figure 21. *E. coli* B transconjugant containing the plasmid pPM21. Plasmid DNA isolated from an *E. coli* B transconjugant following mating with *P. aeruginosa* 27853-1s pPM21. (lane 1 - λ *Hind*III, lane 2 - *P. aeruginosa* 27853-1s pPM21, lane 3 - *E. coli* B transconjugant, lane 4 - *E. coli* B).

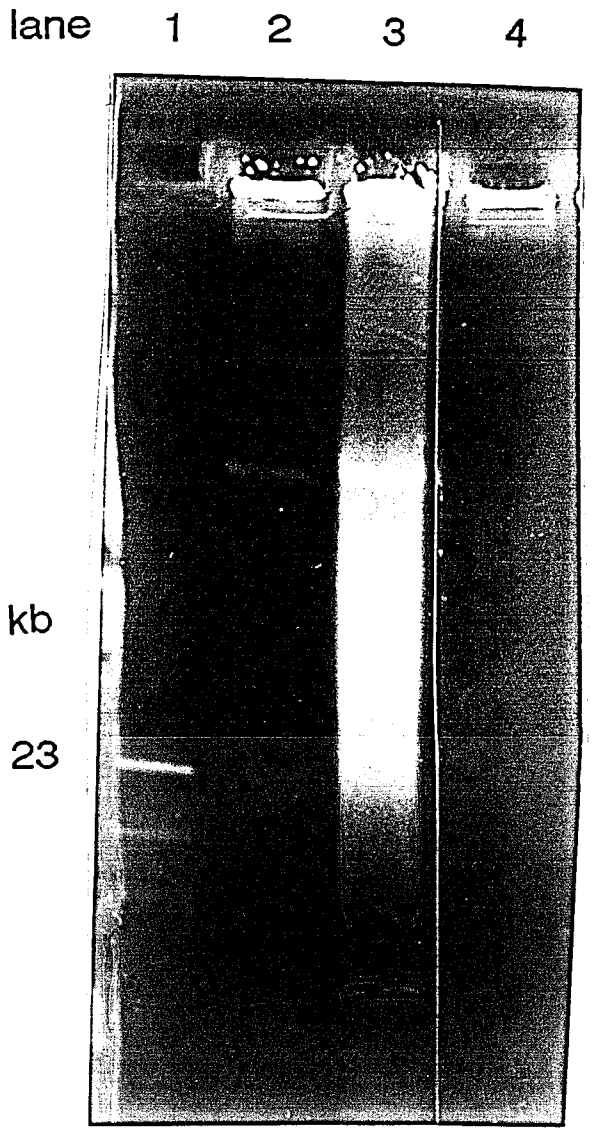

Figure 22. ***E. coli*** **B transconjugant containing the plasmid pPM15.** Plasmid DNA isolated from an *E. coli* B transconjugant following mating with *P. aeruginosa* 27853-1s pPM15. (lane 1 - λ *Hind*III, lane 2 - *P. aeruginosa* 27853-1s pPM15, lane 3 - *E. coli* B transconjugant, lane 4 - *E. coli* B).

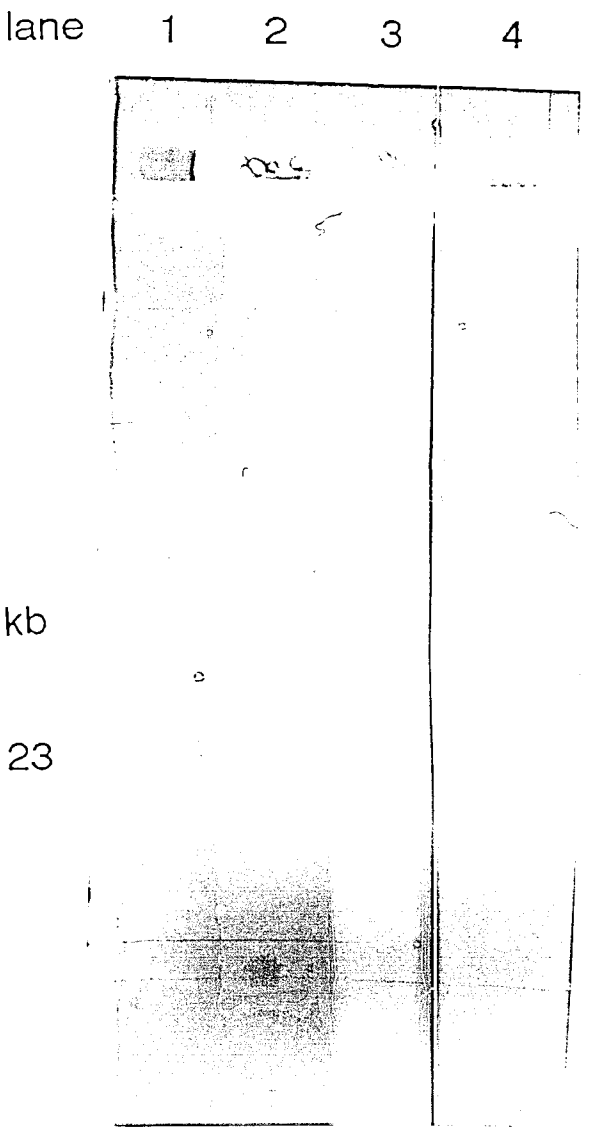

Figure 22. *E. coli* B transconjugant containing the plasmid pPM15. Plasmid DNA isolated from an *E. coli* B transconjugant following mating with *P. aeruginosa* 27853-1s pPM15. (lane 1 - λ *Hind*III, lane 2 - *P. aeruginosa* 27853-1s pPM15, lane 3 - *E. coli* B transconjugant, lane 4 - *E. coli* B).

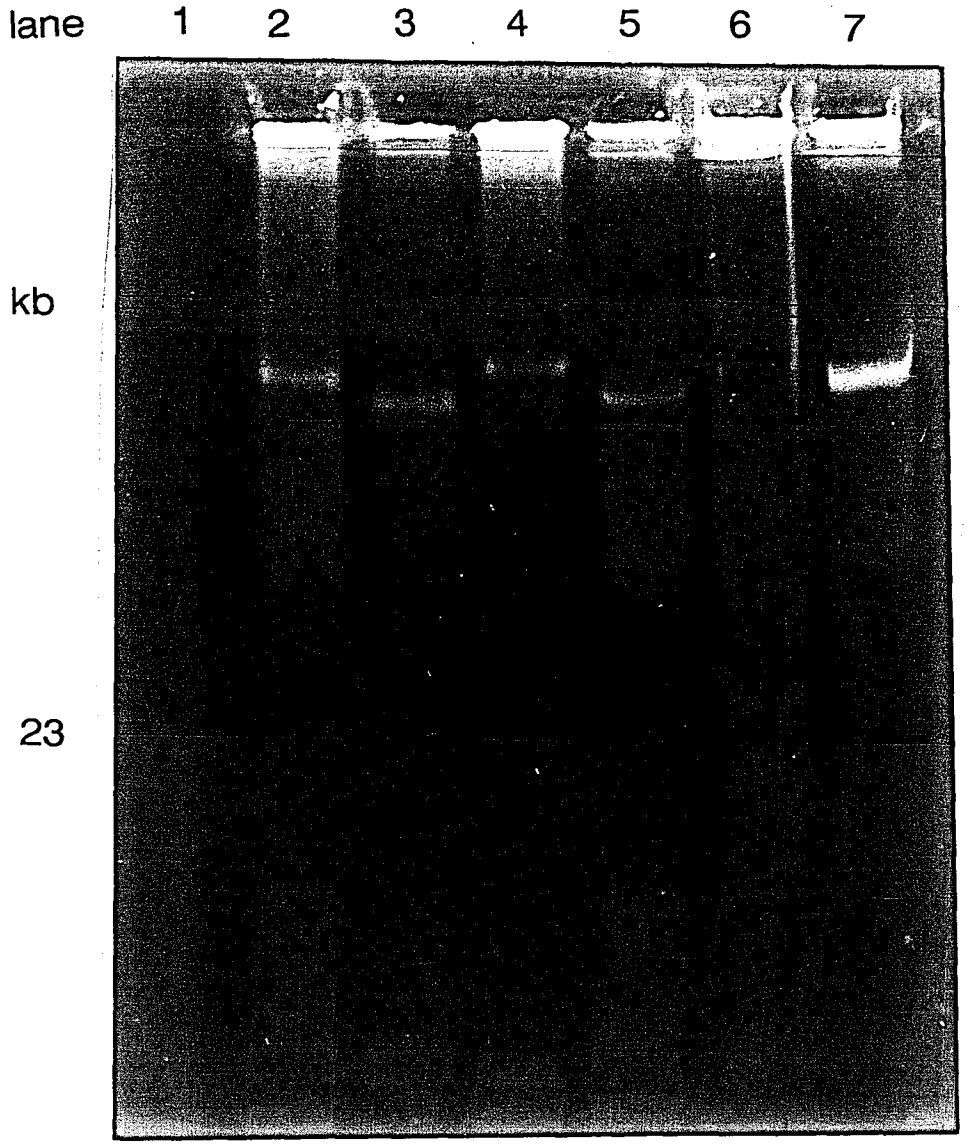

Figure 23. Plasmids isolated from the *E. coli* B transconjugants after storage in the absence of selection as compared to the plasmid isolated from the *P. aeruginosa* met$^+$ transconjugants. Isolated plasmids were electrophoresed through a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. aeruginosa* 11s pPM4, lane 3 - *E. coli* B pPM4, lane 4 *P. aeruginosa* 1s pPM15, lane 5 - *E. coli* B pPM15, lane 6 *P. aeruginosa* 1s pPM21, lane 7 - *E. coli* B pPM21).

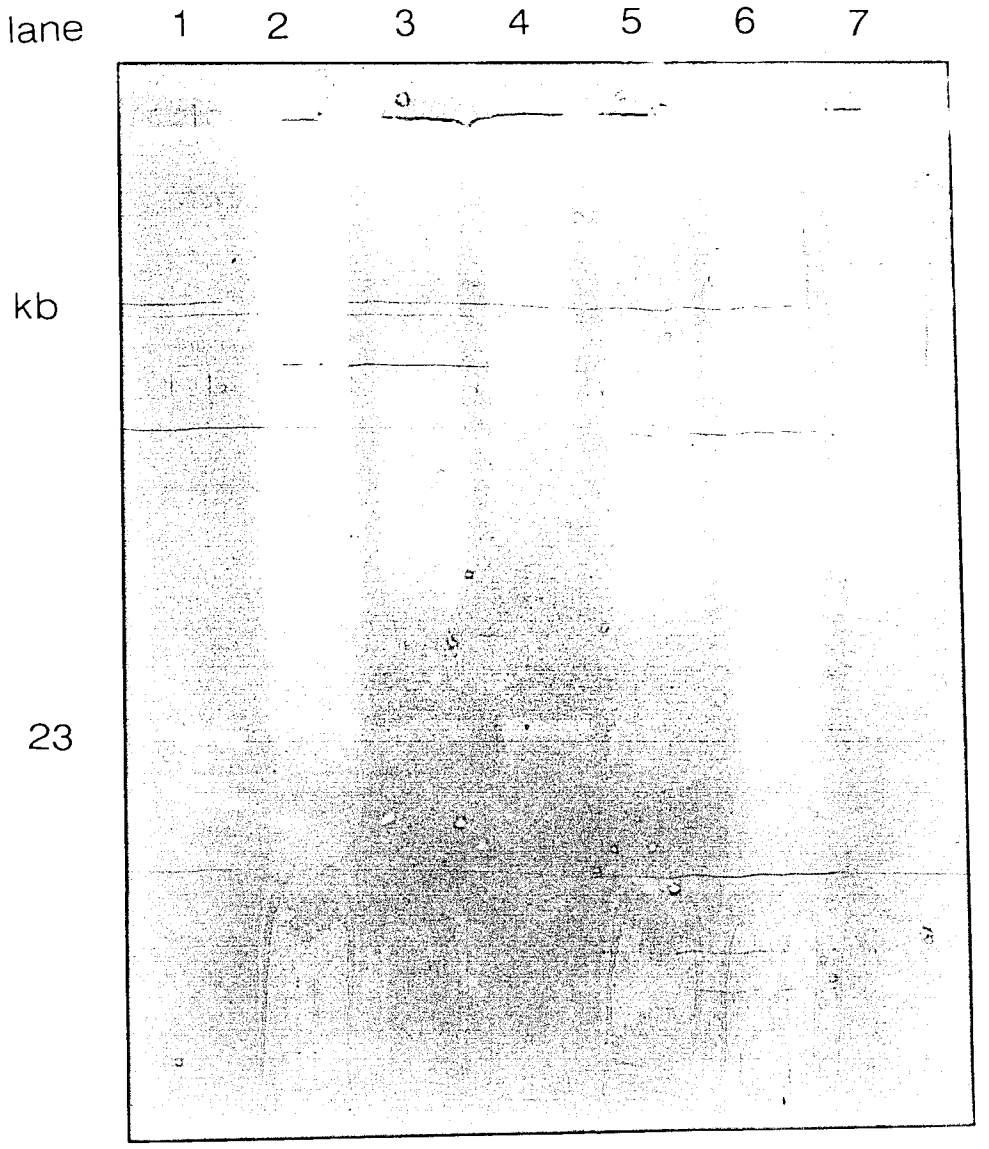

Figure 23. Plasmids isolated from the *E. coli* B transconjugants after storage in the absence of selection as compared to the plasmid isolated from the *P. aeruginosa* met⁻ transconjugants. Isolated plasmids were electrophoresed through a 1% agarose gel. (lane 1 - λ *Hind*III, lane 2 - *P. aeruginosa* 11s pPM4, lane 3 - *E. coli* B pPM4, lane 4 *P. aeruginosa* 1s pPM15, lane 5 - *E. coli* B pPM15, lane 6 *P. aeruginosa* 1s pPM21, lane 7 - *E. coli* B pPM21).

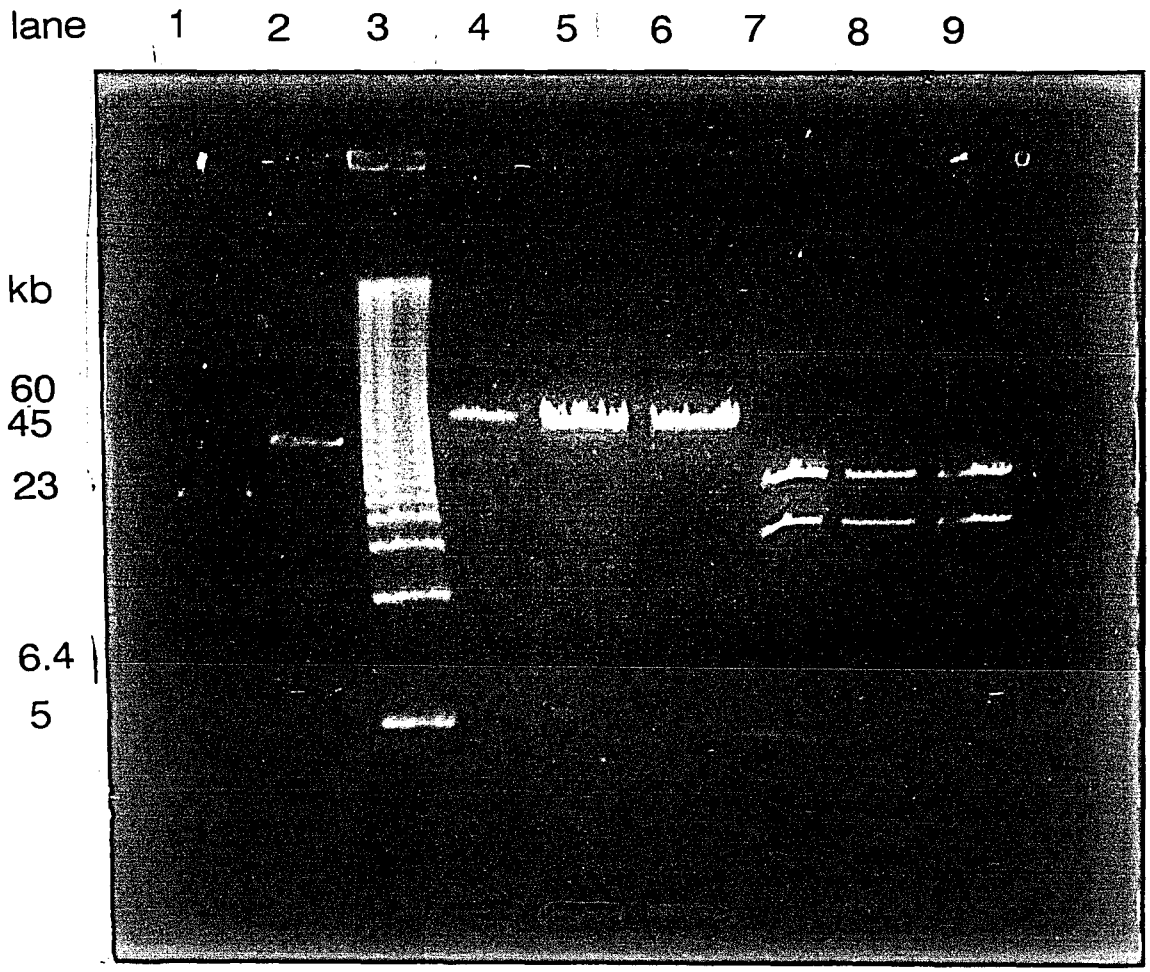

Figure 24. Restriction analysis of pPM21 isolated from *E. coli* B and R68.45 isolated from *P. putida* 2003. Plasmid DNA was restricted with *Eco*RI and double digested with *Sal*I and *Hind*III followed by separation using pulsed field gel electrophoresis. (lane 1 - λ *Hind*III, lane 2 - λ uncut, lane 3 - 5 kb ladder, lane 4 - R68.45 *Eco*RI, lane 5 - pPM21 *Eco*RI, lane 6 - R68.45 and pPM21 *Eco*RI, lane 7 - pPM21 *Sal*I and *Hind*III, lane 8 - R68.45 *Sal*I and *Hind*III, lane 9 - R68.45 and pPM21 *Sal*I and *Hind*III).

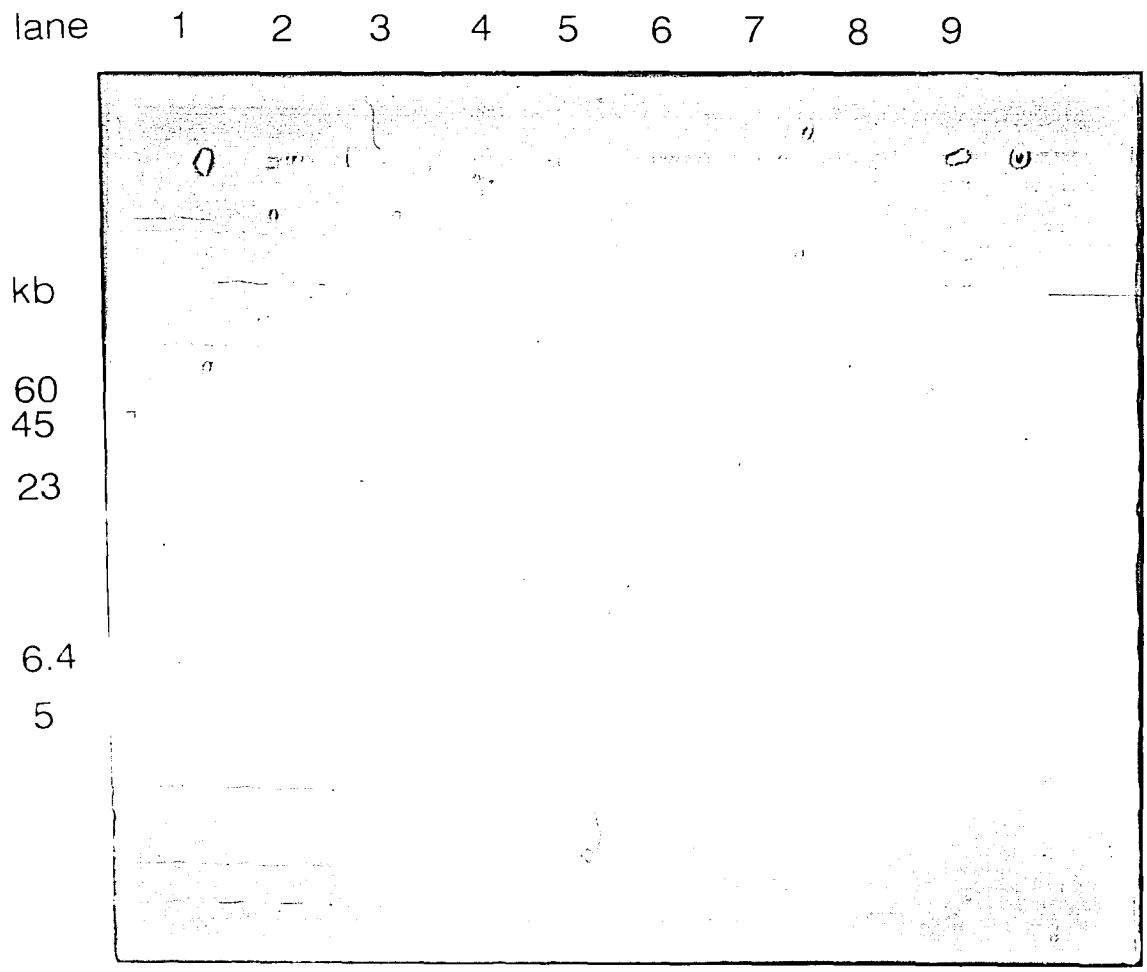

Figure 24. Restriction analysis of pPM21 isolated from *E. coli* B and R68.45 isolated from *P. putida* 2003. Plasmid DNA was restricted with *Eco*RI and double digested with *Sal*I and *Hind*III followed by separation using pulsed field gel electrophoresis. (lane 1 - λ *Hind*III, lane 2 - λ uncut, lane 3 - 5 kb ladder, lane 4 - R68.45 *Eco*RI, lane 5 - pPM21 *Eco*RI, lane 6 - R68.45 and pPM21 *Eco*RI, lane 7 - pPM21 *Sal*I and *Hind*III, lane 8 - R68.45 *Sal*I and *Hind*III, lane 9 - R68.45 and pPM21 *Sal*I and *Hind*III).

Table 1. Bacterial species. Bacterial species used in the experimentation.

Bacterial species	Relevent phenotype	Source
P. putida		
PRS 2003 (R68.45)	$Ben^-, Km^r, Cb^r, Tc^r, 42°C^-$	Dr. M. Shanley
PRS 2003b_2	Km^s, Cb^s, Tc^s	This Study
P. aeruginosa		
27853	$Met^+, Km^r, Cb^s, Tc^s, 42°C^+$	ATCC
27853-1s	$Met^-, Km^r, Cb^s, Tc^s, 42°C^+$	This Laboratory
27853-11s	$Met^-, Km^r, Cb^s, Tc^s, 42°C^+$	This Laboratory
27853-1s (pPM15)	$Met^+, Km^r, Cb^r, Tc^r, 42°C^+$	This Study
27853-1s (pPM21)	$Met^+, Km^r, Cb^r, Tc^r, 42°C^+$	This Study
27853-11s (pPM4)	$Met^+, Km^r, Cb^r, Tc^r, 42°C^+$	This Study
E. coli		
HB101	Km^s, Cb^s, Tc^s	ATCC
HB101 (R68.45)	Km^r, Cb^r, Tc^r	This Study
B "wild type"	Km^s, Cb^s, Tc^s	ATCC
B (pPM15)	Km^r, Cb^r, Tc^s	This Study
B (pPM21)	Km^r, Cb^r, Tc^s	This Study
B (pPM4)	Km^r, Cb^r, Tc^s	This study

Table 2: Biolog data. Biolog data of *P.putida* 2003, *P. aeruginosa* 27853-1s, *P. aeruginosa* 27853-11s, *P. aerurginosa* 27853-1s R68.45, *P. aeruginosa* 27853-11s R68.45, *P. aeruginosa* 27853-1s pPM15, *P. aeruginosa* 27853-1s pPM21, and *P. aeruginosa* 27853-11s pPM4.

Test Organism	ID	Similarity[a]	Distance[b]
P. putida 2003	[c]*P. putida* *P. putida*	0.528 0.762	2.78 2.78
P. aeruginosa 27853-1s	*P. aeruginosa*	0.788	2.53
P. aeruginosa 27853-11s	*P. aeruginosa*	0.721	3.42
P.aeruginosa[d] 27853-1s R68.45	*P. aeruginosa*	0.663 0.747	4.32 3.261
P. aeruginosa 27853-11s R68.45	*P. aeruginosa*	0.587	5.18
P. aeruginosa 27853-1s pPM15	*P. aeruginosa*	0.751	3.418
P. aeruginosa 27853-1s pPM21	*P. aeruginosa*	0.700	3.723
P. aeruginosa 27853-11s pPM4	*P. aeruginosa*	0.777	2.723

a) Similarity describes the probability that the reference organism differs from the unknown organism. A similarity of 0.5 or above is considered a good identification.
b) Distance measures the number of mismatches in the pattern produced by the unknown organism to the reference pattern.
c) Donor for the mating between *P. putida* and *E. coli* HB101.
d) A representative transconjugant from each mating was subjected to biolog analysis.

Table 3: Frequency of R68.45 transfer to *E. coli* HB101. Frequency of transfer of R68.45 from *P. putida* 2003 to *E. coli* HB101.

Number of cells Donor *P. putida* 2003	Number of cells Transconjugants *E. coli* HB101	Frequency of transfer
2.9×10^{7}	3.8×10^{5}	1.31×10^{-2}

Table 4: Frequency of R68.45 transfer and R-prime formation. Frequency of R68.45 transfer and R-prime formation during the mating of *P. putida* 2003 and *P. aeruginosa* 27853-1s/11s.

Number of cells Donor 2003	Number of cells Transconjugant (R68.45) 27853-1s/11s	Number of cells Transconjugant (R-met) 27853-1s/11s	Frequency of transfer	Frequency of R-prime
2.03×10^{8}	* 2.2×10^{7}	* 2.0×10^{2}	1.1×10^{-1}	9.9×10^{-7}
2.5×10^{7}	* 5.5×10^{6}	* 4.2×10^{3}	2.2×10^{-1}	1.7×10^{-4}
6.5×10^{7}	2.4×10^{7}	9.0×10^{1}	3.7×10^{-1}	1.4×10^{-6}

* *P. aeruginosa* 27853-1s

Table 5: Frequency of R-*met* transfer to *E. coli* B.
Frequency of R-*met* transfer from either *P. aeruginosa* 27853-1s or *P. aeruginosa* 27853-11s to *E. coli* B.

Number of cells Donor	Number of cells Transconjugants *E. coli* B	Frequency of transfer
27853-1s pPM15 2.7×10^{8}	1.9×10^{5}	7.0×10^{-4}
27853-1s pPM21 5.0×10^{7}	3.1×10^{4}	6.2×10^{-4}
27853-11s pPM4 3.1×10^{8}	1.2×10^{4}	3.9×10^{-5}

Table 6: Transfer of carbenicillin resistance and methionine prototrophy by pPM4, pPM15, and pPM21. Frequency of plasmid marker transfer (carbenicillin resistance) and frequency of chromomal marker (*met* determinant) transfer during the mating between the *E. coli* B transconjugants containing the possible R-*met* primes and the *P. aeruginosa* methionine auxotrophs.

Number of cells Donor	Number of cells Transconjugants Cb^r	Number of cells Transconjugants met^+	Frequency of Cb^r transfer	Frequency of met^+ transfer
E. coli B pPM4 1.4×10^8	*P. aeruginosa* 27853-11s 2.3×10^5	*P. aeruginosa* 27853-11s 1.5×10^3	1.6×10^{-3}	1.1×10^{-5}
E. coli B pPM15 4.4×10^8	*P. aeruginosa* 27853-1s 1.0×10^5	*P. aeruginosa* 27853-1s 2.9×10^2	2.3×10^{-4}	6.6×10^{-7}
E. coli B pPM21 4.7×10^8	*P. aeruginosa* 27853-1s 3.3×10^4	*P. aeruginosa* 27853-1s 1.1×10^2	7.0×10^{-5}	2.3×10^{-7}

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Patricia A. McQueney

Curriculum Vitae

Department of Molecular Biology
113 Williams Hall
Lehigh University
Bethlehem, PA 18015
(215) 758-5175

313 Cherokee St
Bethlehem, PA 18015
(215) 861-2937

EDUCATION:

M.S. Molecular Biology, December, 1991
Lehigh University
Advisor: Professor Steven Krawiec
Thesis entitled: Analysis of R68.45 and R68.45-*met* R-primes by Physical and Genetic Criteria.

B.S. Biology, December, 1988
Millersville University, Millersville PA

PROFESSIONAL EXPERIENCE:

1/90 - present	**Research fellow**, Department of Molecular Biology, Lehigh University
8/89 - 12/89	**Teaching Assistant**, Department of Biology, Lehigh University Taught Humanistic Botany
2/29 - 8/89	**Biological Manufacturing Technician**, Wyeth-Ayerst, Marietta PA Produced the influenza virus vaccine

Honors:

1/90 - present	Recipient of a National Institutes of Health Biotechnology Training Fellowship
1/90	Selected as an Outstanding Young Woman of America
12/88	Graduated with magna cum laude honors
9/87 - present	Member of the Phi Kappa Phi National Honor Society

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